REACTIONS OF AQUEOUS CHLORINE AND CHLORINE DIOXIDE WITH L-TRYPTOPHAN: GENOTOXICITY STUDIES AND IDENTIFICATION OF SOME GENOTOXIC REACTION PRODUCTS

BY

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Aqueous chlorine and chlorine dioxide reacted readily with tryptophan at three different molar ratios (1:1, 3:1 and 7:1 disinfectant:tryptophan) to produce complex reaction mixtures and a dark precipitate. Evaluation of the mutagenic activity of the reaction products using the Ames Salmonella/ microsome assay indicated that they were mutagenic at all the three ratios used. The nonvolatile mutagenic reaction products were mainly acidic and neutral compounds; they were direct-acting and caused both frameshift and base-pair substitution mutations. The reaction products were also capable of increasing the sister chromatid exchange frequencies in vitro in Chinese hamster ovary cells.

Amberlite XAD polymeric resin adsorption was superior to liquid-liquid extraction for concentrating small quantities of organic mutagens from the model reaction mixtures.

At the lower disinfectant doses (1:1 and 3:1), the reaction products of chlorine dioxide with tryptophan were either more potent mutagens or were similar to that of aqueous chlorine; while at the highest ratio (7:1), aqueous chlorine reaction products were consistently more mutagenic. Thin layer chromatographic separation of the reaction products and detection under ultraviolet light revealed the presence of several blue and green fluorescent compounds which had not been previously reported. The use of radiolabeled ^{14}C-tryptophan indicated the most potent mutagens were produced in very minute quantities and represented less than 1% of the total starting material. Gas chromatography/mass spectrometry of the reaction products at the 1:1 molar ratio did not reveal the presence of any chlorinated compounds. The compounds identified at this ratio included isatin, oxindole and indolylacetonitrile. When an excess of chlorine (7:1 molar) was used, several chlorinated compounds were identified. Chloral, 1,1,3,3-tetrachloropropanone and 1,1,3-trichloropropanone are some of the known mutagens and chloroform precursors identified from these reactions.

CHAPTER I
INTRODUCTION

Background

Chlorine is used on a worldwide basis as a disinfectant for potable water and for treatment of sewage effluents. It is also used as an anti-foulant in electric plants and as a biocide for the treatment of industrial waters and in food processing (White, 1972; Morris, 1971). The commercial use of chlorine has greatly improved the quality of life and prevented the transmission of water-borne diseases throughout the world. In the food industry, chlorine is intentionally added to flour for oxidizing and bleaching purposes, and to cooling and cleaning waters for milk, fish, meats, poultry and fruits and vegetables (Smith and Arends, 1984; Foegeding, 1983; Kotula et al., 1974; Ranken et al., 1965; Mercer and Sommers, 1957). Dilute aqueous chlorine solutions may be used to decontaminate fruits, eggs and vegetables (Dychdala, 1983). Chlorine is FDA approved and may also come into contact with foods unintentionally when it is used as a cleaning and sterilizing agent for food processing equipment and packaging.

Concern about the use of chlorine as a disinfectant for potable water arose when it was discovered that

trihalomethanes (THM) are produced as undesirable side products as a result of water chlorination practices (Bellar et al., 1974; Rook, 1974). These concerns were exacerbated when the National Cancer Institute confirmed that chloroform, a major trihalomethane, was carcinogenic in laboratory animals (NCI, 1976). This led the U.S. Environmental Protection Agency to make an amendment to the National Interim Primary Drinking Water Regulations that set a maximum contaminant level of 0.10 mg/L for total THMs (USEPA, 1983). Since this revelation, other halogenated and non-halogenated byproducts have been shown to be produced as a result of water chlorination practices (Coleman et al., 1984; Christman et al., 1983; Loper et al., 1978; Rook, 1977). In addition to chloroform, other halogenated byproducts such as the di- and trichloroacetonitriles and their brominated analogs which are produced during water chlorination, have also been shown to be genotoxic (Bull et al., 1986; Daniel et al., 1986; Weisburger, 1977).

Free, proteinaceous and bound amino acids as well as fulvic and humic substances present in potable water have been implicated and confirmed as precursors of chlorination reaction products (Bierber and Trehy, 1983; Oliver, 1983; Lytle and Perdue, 1981; Trehy and Bierber, 1981; Christman, 1980). In fact, extracts of chlorinated drinking water, amino acids and fulvic and humic substances have been shown to be genotoxic in different short-term assays (Horth et al., 1987;

Tan et al., 1987b; Fielding and Horth, 1986; Bull and Robinson, 1985; Meier et al., 1983; Sussmuth, 1982; Nazar and Rapson, 1982; Loper, 1980; Nestmann et al., 1980). The difficulty encountered in the interpretation of these findings is that other poorly characterized compounds are also present which may or may not contribute to the observed genotoxicity. Epidemiological evidence has also been adduced to link certain cancers of the colon, bladder and rectum to water chlorination practices (Cantor et al., 1985; Cragle et al., 1985; Crump, 1983; Williamson, 1981; Cantor and McCage, 1978).

The formation of undesirable toxic side products during chlorination to produce potable water and the need to reduce total THM levels in drinking water to below 0.10 mg/L, has led to a search for alternate disinfectants (USEPA, 1983). Chlorine dioxide has been proposed as an alternative oxidant and drinking water disinfectant in America and has received numerous favorable reviews (Knocke et al., 1987; Lauer et al., 1986; Lykins and Griese, 1986; Masschelein, 1985; Aieta et al., 1980). This is because, unlike chlorine, chlorine dioxide produces very little or no THMs in treated water and it does not react with ammonia commonly found in water to form chloramines (Federal Register, 1983; Symons et al., 1981; White, 1972). Even though chlorine dioxide produces much fewer THMs, its use is limited by cost (about 3 times that of chlorine) and the possible health hazards associated with its biological imapct and long-term use which are not known.

Objectives of the Study

Amino acids are ubiquitous in the environment and have been shown to act as precursors of mutagenic and/or carcinogenic byproducts in drinking water. Based on the reactions of chlorine and/or chlorine dioxide with amino acids, the following general objectives were proposed for this study:

1. To investigate the mutagenic potential of the reaction products of the environmentally significant amino acid, tryptophan, with different molar concentrations of aqueous chlorine or chlorine dioxide using available concentration techniques and the Ames Salmonella mutagenicity assay.
2. To compare liquid-liquid extraction and Amberlite XAD adsorption for concentrating the reaction products and also to compare the mutagenic activities of aqueous chlorine and chlorine dioxide reaction products.
3. To separate the reaction products using different chromatographic methods and to re-evaluate the mutagenicity of the fractions using the Ames Salmonella/microsome assay. In addition, to investigate the highly mutagenic subfractions for genotoxicity using the sister chromatid exchange assay in cultured Chinese hamster ovary cells.

4. To identify the reaction products in the highly mutagenic fractions and propose mechanisms for their formation.

CHAPTER II
LITERATURE REVIEW

Historical Perspectives

Chlorine is an member of the halogen family and although it is one of the most widely distributed elements on earth, it is never found uncombined in nature. It exists in the earth's crust in the form of soluble salts of potassium, sodium, calcium and magnesium (Dychdala, 1983; White, 1972).

Chlorine is believed to have been known to medieval chemists long before it was discovered in its gaseous state in 1774 by a Swedish chemist named Steele (Baldwin, 1927). Steele called the gas dephlogisticated muriatic acid and observed that it was soluble in water and had permanent bleaching effects on paper, vegetables and flowers. The gas was liquefied by compression in 1805 and was found to act on metals and their oxides. Davy declared the gas to be an element and named it chlorine, derived from the Greek word "chlorous" which has been translated to mean green, greenish yellow or yellowish green, based on its color (Baldwin, 1927). In 1811 Davy discovered chlorine dioxide gas when he poured a strong acid on sodium chlorate (White, 1972). He observed that the gas had an intense color similar to that of chlorine and subsequently named it euchlorine.

Even though the bleaching action of chlorine had been used for a long time, the disinfecting and deodorizing properties were not known until the first part of the nineteenth century. Chlorinated lime was used for disinfecting and deodorizing hospitals and for sewage treatment in London in 1854 (Dychdala, 1983). In 1881, Koch demonstrated the bactericidal action of chlorine as hypochlorite on pure bacterial cultures under controlled laboratory conditions and in 1886, the American Public Health Association issued a favorable report on the use of hypochlorites as disinfectants. In 1894, Traube established the first guidelines for hypochlorites in water purification and disinfection (Dychdala, 1983; Hadfield, 1957). Chloride of lime was first introduced to north America for water treatment in the early part of the nineteenth century and within a few years, over 800 million gallons of water had been purified by chlorination (Dychdala, 1983; Race, 1918). To date, chlorine has gained worldwide recognition as the disinfectant of choice for the treatment of potable water.

The use of chlorine in medicine began in 1915 when Dakin used dilute solutions of sodium hypochlorite for disinfecting open and infected wounds during World War I. Dakin's original formulation contained chlorinated lime, sodium carbonate and boric acid (Dychdala, 1983). The dairy industry was perhaps the first food processing industry to utilize the disinfecting and deodorizing properties of chlorine (Mercer and Sommers,

1957). In this respect chlorine was used as a sanitizer for milk bottles and milk equipment. Use of chlorine then spread to other food industries and it is used in all branches of food processing including canning, flour, poultry, fish, meat and fruits and vegetables industries.

Chemistry of Aqueous Chlorine

In water, chlorine gas undergoes rapid hydrolysis to form hypochlorous acid according to the following equation (Morris, 1946):

$$Cl_2\ (g) + H_2O \longrightarrow H^+ + Cl^- + HOCl \qquad 1.$$

A small amount of a id , H^+, is produced; however, it tends to lower the pH of the solution. Other molecular and ionic species such as H_2OCl^+, OCl^- and Cl_2 are produced; however, HOCl is by far the most reactive (Morris, 1978). Consistent with industrial practice, the term chlorine will be used in this text to refer to aqueous solutions of the active chlorine compounds, OCl^-, Cl_2, and HOCl. Molecular chlorine gas will be referred to as such or as chlorine gas. The active chlorine compounds, OCl^-, Cl_2 and HOCl coexist in solution and are difficult to distinguish at the trace level, therefore, they are grouped together as free residual chlorine (White, 1972).

Hypochlorous acid is classified as a weak acid which tends to undergo partial dissociation as follows:

$$HOCl \longrightarrow H^+ + OCl^- \qquad 2.$$

The dissociation constant for equation 2 ranges from 1.6 to 3.2 x 10^{-8} for the temperature range 0-25°C (Morris, 1966). The amounts and ratios of the different ionic and molecular species present at any particular time are dependent upon pH, temperature and chlorinity or dissolved solids. For example, at pH 6.0 and a temperature of 15°C, the fractions of HOCl, Cl_2 and OCl^- are 0.975, 3.6 x 10^{-5} and 0.025, respectively. However, when the pH is increased to 9.0 under identical conditions, the fractions become 0.038, 1 x 10^{-9} and 0.962, respectively (Morris, 1978).

The above-mentioned aqueous chlorine species have different reactivities which are related to their redox potentials as follows (Masschelein, 1979):

$$Cl_2 + 2e^- \longrightarrow 2Cl^- \quad (1.36V) \qquad 3.$$

$$HOCl + H^+ + 2e^- \longrightarrow Cl^- + H_2O \quad (1.49V) \qquad 4.$$

$$OCl^- + H_2O + 2e^- \longrightarrow Cl^- + 2OH^- \quad (0.9V) \qquad 5.$$

Of the three species, HOCl is by far the most reactive in the pH range of 6-7. Morris (1978) has shown that towards nitrogenous substrates, HOCl is 300, 800 and 4 x 10^4 more reactive than Cl_2, H_2OCl^+, and OCl^-, respectively, in aqueous solutions at 15°C and neutral pH.

Reaction with inorganics

Due to its reactivity, chlorine or hypochlorous acid readily oxidizes all organic and inorganic materials in aqueous solutions until at some "breakpoint," when demand is fully satisfied (Dychdala, 1983). Inorganic substances such

as carbon, cyanide, iron, nitrite, sulfite, sulfide and manganese react with hypochlorous acid via oxidation. Either the chlorine atom or the oxygen atom of hypochlorous acid may act as the center of reaction. The chlorine atom is more electropositive, therefore, electrophilic reactions proceed by way of the chlorine atom (Morris, 1978).

Chlorine reacts rapidly with inorganic carbon according to the following expression:

$$C + 2Cl_2 + 2H_2O \longrightarrow 4HCl + CO_2 \qquad 6.$$

The above reaction takes place in dechlorination processes which usually employ granular carbon filters, and the acid formed consumes alkalinity in treated waters (White, 1972).

Reducing substances. Hydrogen sulfide, characterized by its obnoxious odors, is a constituent of septic sewage and is commonly found dissolved in underground waters. During drinking water chlorination, hydrogen sulfide is completely oxidized to sulfate according to equation 7 (White, 1972):

$$H_2S + 4Cl_2 + 4H_2O \longrightarrow H_2SO_4 + 8HCl \qquad 7.$$

Nitrites are oxidized readily by free available chlorine to produce nitrates in a reaction similar to the above (Huburt, 1934):

$$NO_2^- + HOCl \longrightarrow NO_3^- + H^+ + Cl^- \qquad 8.$$

Chlorine reacts with iron in water supplies to remove iron from water and to produce a coagulant for both water and sewage treatment. Similarly, under alkaline conditions, chlorine reacts with cyanide to form cyanate and with soluble

manganese compounds to produce insoluble manganese dioxide which can be removed by filtration. The cyanide process represents a detoxication mechanism in water treatment operations (White, 1972).

Ammonia. The reaction of chlorine with ammonia in dilute aqueous solutions is of practical importance in water treatment and has been shown to produce three types of N-chloro compounds or chloramines. In these reactions, the electrophilic chlorine atom from HOCl attaches itself to the bare electron pair of the nitrogen atom with the concurrent release of H^+ from ammonia and OH^- from HOCl to rapidly form monochloramine and water as shown in equation 9 (Morris, 1978). The reaction can proceed slowly to form dichloramine and even more slowly to produce trichloramine or nitrogen trichloride according to the following equations:

$$HOCl + NH_3 \longrightarrow NH_2Cl + H_2O \qquad 9.$$

$$HOCl + NH_2Cl \longrightarrow NHCl_2 + H_2O \qquad 10.$$

$$HOCl + NHCl_2 \longrightarrow NCl_3 + H_2O \qquad 11.$$

The reactions occur in sequence and, therefore, compete with each other. The chloramines possess some oxidative power and are collectively referred to as combined residual chlorine. Factors affecting the types and ratios of chloramines formed include pH, temperature, contact time and initial chlorine to ammonia ratio (White, 1972). For example, equation 9 will rapidly convert all the free chlorine to monochloramine at pH 7-8 when the ratio of chlorine to ammonia is equimolar, i.e.,

5:1 by weight or less. When the molar ratio of chlorine to ammonia is increased, products from equations 10 and 11 can also be formed depending on the conditions (White, 1972).

Equations 10 and 11 are related to the "breakpoint" phenomenon used in water treatment for the control of obnoxious tastes and odors. "Breakpoint" chlorination refers to the use of excess amounts of chlorine beyond the water demand, thus resulting in residual free available chlorine (Griffin, 1946). Breakpoint chlorination leads to increased germicidal efficiency and disappearance of foul tastes and odors naturally present in water. At or above the breakpoint, the initial monochloramine starts a disproportionation reaction to form dichloramine according to equation 10, which in turn forms trichloramine or nitrogen trichloride as the chlorine residuals increase further (White, 1972; Griffin, 1944; Griffin and Chamberlin, 1941).

Other halogens. Apart from chlorine, bromine is perhaps the second most important halogen with regard to water chlorination. Bromide, a constituent of natural waters and seawater, reacts rapidly with chlorine or hypochlorite according equation 12:

$$Br^- + HOCl \longrightarrow HOBr + Cl^- \qquad 12.$$

The HOBr is a strong electrophile and reacts much more rapidly than HOCl (Morris, 1978). A primary difference between bromine and chlorine systems is that at neutral pH there are higher concentrations of molecular bromine, Br_2, than

molecular chlorine. Also, at any given pH, HOBr ionizes much more rapidly than HOCl (Jolley and Carpenter, 1983). After reaction with organics, HOBr is reduced to Br^- which is reoxidized to HOBr by residual aqueous chlorine in the water. The overall result is enhanced reactivity of aqueous chlorine in the presence of the bromide ion (Morris, 1978).

Upon chlorination of natural waters containing iodide, chlorine readily oxidizes the iodide according to the following equation which forms the basis of the iodometric method for chlorine determination (Jolley and Carpenter, 1983; Lister and Rosenblum, 1963).

$$HOCl + 2I^- \longrightarrow I_2 + Cl^- + OH^- \qquad 13.$$

Molecular iodine hydrolyses rapidly to form hypoiodous acid which undergoes dissociation according to equation 14:

$$HOI \longrightarrow H^+ + OI^- \qquad 14.$$

Equation 14 becomes important only at high pH and is not of practical significance in the chlorination of either natural or wastewater systems (Jolley and Carpenter, 1983).

Reaction with organics

Reaction of chlorine with organics in drinking water proceeds via oxidative degradation or chlorine incorporation through electrophilic substitution or addition reactions. The major known products of the latter reactions are THMs whose identification in treated water aroused serious health concerns in the United States and elsewhere (Bellar, et al., 1974; Rook, 1974).

Oxidation. The predominant type of reaction involving chlorine and organic compounds in natural and process waters is oxidation. Carbohydrates and related compounds constitute a class of organic compounds likely to undergo oxidation reactions in aqueous solutions (Jolley et al., 1978; Whistler et al., 1966). Heterocyclic aromatic compounds such as the purines, pyrimidines and indoles have been shown to undergo oxidative reactions in dilute aqueous chlorine solutions (Lin and Carlson, 1984; Hoyano et al., 1973). Indole has been shown to yield oxindole and isatin upon reaction with chlorine in dilute aqueous solutions (Lin and Carlson, 1984).

Other classes of compounds known to undergo oxidative reactions with aqueous chlorine or hypochlorite solutions include proteins and amino acids, sulfur-containing compounds, lipids, and polynuclear aromatic hydrocarbons (Trehy et al., 1986; Lin and Carlson, 1984; Oyler et al., 1983; Dychdala, 1983; Ghanbari et al,, 1983; Burleson et al., 1980).

Addition. Addition of chlorine or halogens to organic compounds may occur by way of reactive double bonds. This type of reaction is another example of electrophilic attack in which the rate determining step is believed to be transfer of Cl^+ to the double bond to give a chloronium ion (Morris, 1978). Olefins and acetylenes represent a class of compounds that are vulnerable to attack by chlorine. Their major reaction with chlorine is by addition in which chlorine atoms and hydroxyl groups are added to the double bonds (Ghanbari

et al., 1983; Carlson and Caple, 1978; Jolley et al., 1978; Morris, 1978; Pitt et al., 1975; Leopold and Mutton, 1959). For example, aqueous chlorination of oleic acid has been shown to produce a mixture of chlorohydrins with water playing a role in product formation (Carlson and Caple, 1978). Chlorine addition to double bonds is a slow process and proceeds only when the double bonds involved are activated (Morris, 1978).

Substitution. The two major types of substitution reactions of chlorine and chlorine compounds involve formation of either N- or C-chlorinated compounds.

Formation of N-chlorinated compounds. Nitrogen appears as a natural component of foods and other substances either as organic or inorganic nitrogen. Since inorganic nitrogen has been mentioned earlier, the following discussion will focus only on organic nitrogen. Sources of organic nitrogen in natural and process waters include amides, amino acids, indoles, proteins, nucleic acid bases, and their derivatives as well as pesticides (Jolley et al., 1978). Organic nitrogen in water exerts a chlorine demand and leads to the formation of N-chlorinated compounds (White, 1972; Morris, 1967).

The major reaction mechanisms involved in the formation of N-chlorinated compounds have been discussed by Morris (1967). With amines, aqueous chlorine reacts to form N-chloro compounds by a mechanism similar to its reaction with ammonia. The electrophilic chlorine atom attaches itself to the bare electron pair of nitrogen, thus releasing water. Conversely,

the reaction may be viewed as one of displacement whereby the nucleophilic amine displaces OH^- from HOCl (Morris, 1978). Amides also appear to react via the same pattern or via the haloform reaction but at much slower rates. A simplified scheme of chlorine substitution with organic N-substituted organic compounds is shown below (Morris, 1967):

$$R\text{-}NH_2 + HOCl \longrightarrow R\text{-}NHCl + H_2O \quad 15.$$

$$R\text{-}CO\text{-}NH_2 + HOCl \longrightarrow R\text{-}CO\text{-}NHCl + H_2O \quad 16.$$

In both cases, the rates of chloramination vary proportionately with the basic strength or nucleophilicity of the nitrogenous substrate (Morris, 1978). Proteins are more complex than amines and amides and chlorination of organic nitrogen from proteinaceous material is a slow and tedious process. However, proteins may also undergo substitution with chlorine to form N-chlorinated proteinaceous byproducts (Jolley et al., 1978; White, 1972).

Formation of C-chlorinated compounds. Formation of C-chlorinated compounds via chlorine substitution is perhaps the most widespread in nature (Jolley et al., 1978; Morris, 1975; Carlson et al., 1975). The major substitution reactions of chlorine involve substitution into aromatic or heterocyclic compounds and the haloform reaction. Humic and fulvic substances abound in nature and represent vegetation under varying degrees of decay. These complex and otherwise poorly characterized high molecular weight materials contain aromatic and heterocyclic moeities which readily undergo chlorination

reactions in aqueous chlorine solutions when activated (Jolley et al., 1978).

Some substitution reactions involving phenolic compounds and aromatic organic acids are presented in Figure 1. As before, the mechanism of the reaction involves an electrophilic attack of HOCl on the phenoxide ion. In this case the reaction is facilitated by the presence of a negative charge on the nucleophilic substrate and only when the ring is "activated" by substituents such as the oxide ion, does substitution occur (Morris, 1978). Phenols are more reactive in dilute aqueous chlorine solutions than other aromatic compounds possessing different substituent groups (Carlson and Caple, 1978). Substitution may proceed at the activated ortho or para positions not fully occupied by chlorine atoms. Further rupture of the aromatic ring may occur by mechanisms that are not fully understood (Morris, 1978). Heterocyclic aromatic compounds such as the nucleic acid bases may also undergo substitution reactions in which they are either activated or inactivated toward electrophilic attack compared to benzene (Morris, 1978). An example of the activation process involves the chlorination of some purines and pyrimidines. Aqueous chlorination of the pyrimidines, cytosine and uracil has been shown to lead to the formation of their corresponding 5-chloro analogs (Figure 2) and other poorly characterized byproducts (Jolley et al., 1975; Hoyano et al., 1973; Patton et al., 1972).

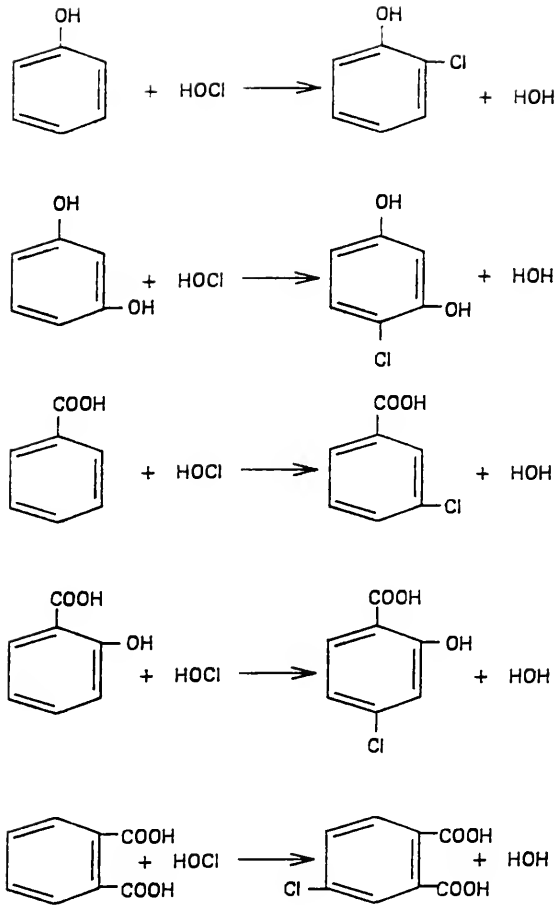

Figure 1. Chlorine substitution reactions with some phenolic compounds and aromatic organic acids (Adapted from Jolley et al., 1978).

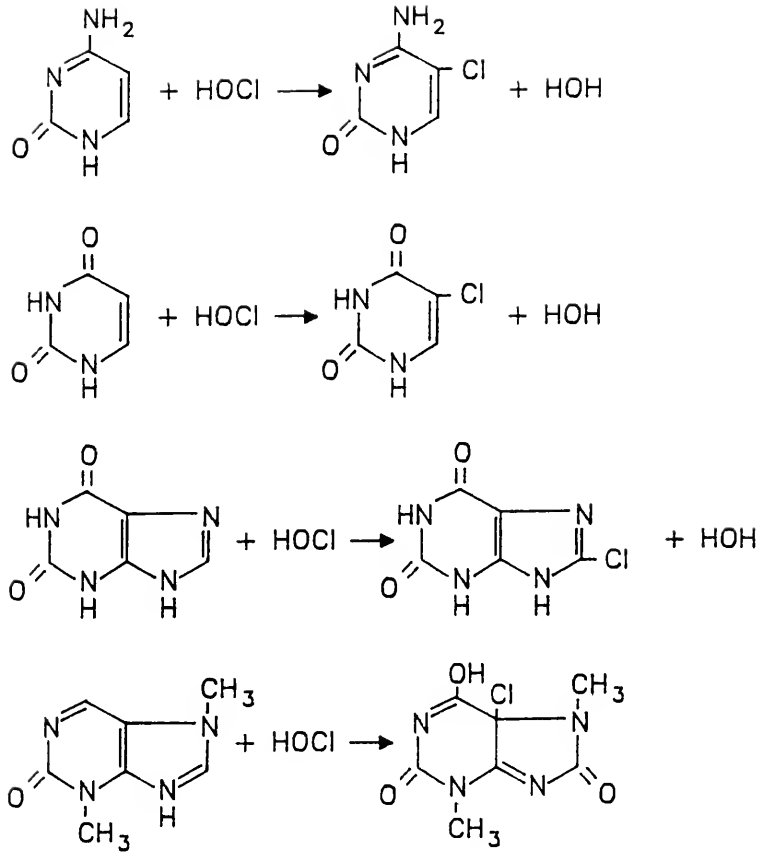

Figure 2. Chlorine substitution reactions with pyrimidines and purines (Adapted from Jolley et al., 1978).

Similar chlorinated products may be obtained with purines as shown in Figure 2. With the purines, actual formation of the chlorinated analogs has not been demonstrated in dilute aqueous chlorine solutions but rather in non-polar solvents and in acetic acid (Jolley et al., 1978). Sources of nucleic acid bases in potable water are plankton, bacteria, and plant and animal matter. Like the nucleic acid bases, pyrroles are also thought to be greatly activated to undergo chlorine substitution reactions (Morris, 1978). Pyridines on the other hand are thought to be sufficiently deactivated so that reaction with dilute aqueous chlorine solutions is not expected to occur in the ring (Morris, 1978).

Finally, formation of C-chlorinated compounds may occur through the haloform reaction (Figure 3) with humic material leading to the formation of volatile compounds such as chloroform (Morris, 1975; Rook, 1974).

Chlorine disinfection

Despite much research done in this area, the actual mechanism of germicidal action of chlorine has not been fully elucidated. It is well known that even in trace amounts, chlorine has fast killing action on bacteria and several theories have been proposed to explain this killing action. To date, none of the theories has been fully accepted due to a lack of substantive evidence (Dychdala, 1983; White, 1972). The proposal that the germicidal action of chlorine is due to the liberation of nascent oxygen held for some time until it

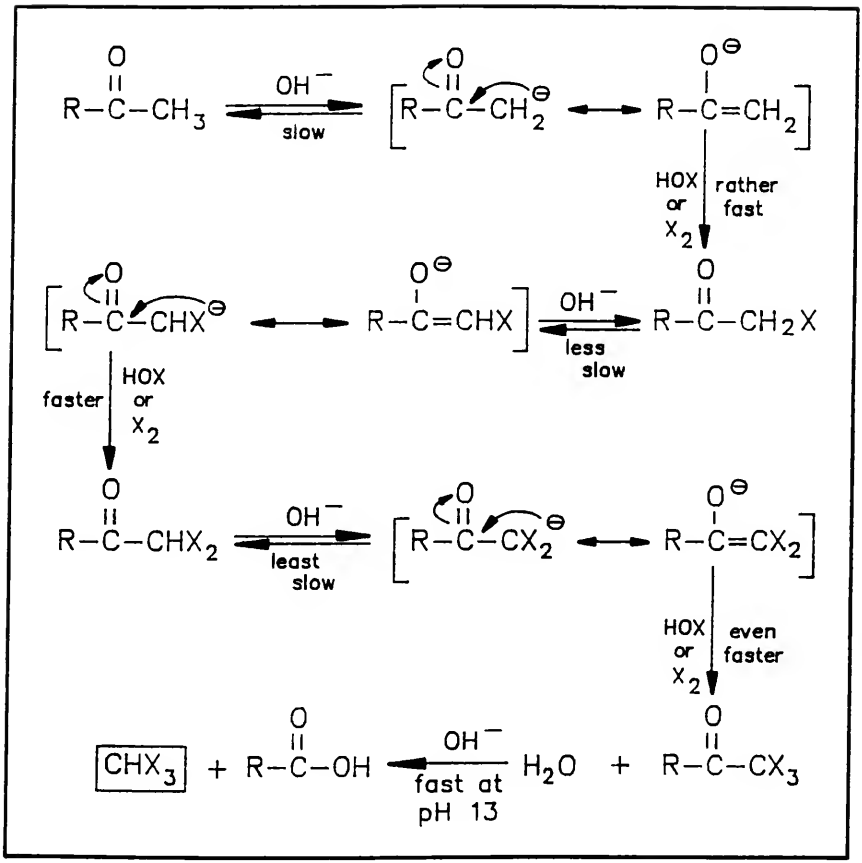

Figure 3. Detailed reaction mechanism of the haloform reaction. X=Halogen (Adapted from Morris, 1985).

was finally dispelled. This is because other strong nascent oxygen producers such as hydrogen peroxide and potassium permanganate are less effective bactericides than chlorine (White, 1972). Also, chlorine still possesses germicidal activity under conditions which exclude direct oxidation of bacterial protoplasm (Mercer and Somers, 1957). Other theories, even though attractive, have not received the full support of researchers in this area. Baker (1926) proposed that chlorine reacted with bacteria cell membrane proteins to produce N-chloro compounds which interfere with bacterial cell metabolism leading to death. The N-chloro theory was taken a step further by Rudolph and Levine (1941) who concluded that the bactericidal activity of chlorine or hypochlorites proceeds in two stages: 1) the penetration of an active ingredient into the bacterial cell, and 2) the chemical reaction of this ingredient with the cellular protoplasm to form toxic N-chloro compounds which destroy the organism. Mercer and Somers (1957) stated that bacterial death by chlorine is a poisoning process in which a form of chlorine combines chemically with the protoplasm of the bacterial cell to produce toxic organic complexes.

Other proposed mechanisms of chlorine disinfection were directed at key enzymes in the target organisms. Green and Stumpf (1946) and later Knox et al. (1948) observed experimentally that there was a correlation between the effect of chlorine on bacterial growth and its effect on the rate of

glucose oxidation by certain bacteria. They were able to show that a bacterial suspension became sterile when the organisms lost their ability to oxidize glucose. The mechanism of inhibition was thought to involve irreversible oxidation of the sulfhydryl groups of essential enzymes like triosephosphate dehydrogenase. The sulfhydryl oxidation theory received additional support when chlorine was shown to oxidize sulfhydryl radicals to sulfonyl compounds and sulfides to sulfates under conditions that simulate water chlorination (Ingols et al., 1953; Black and Goodson, 1952; Douglas and Johnson, 1938). Wyss (1962) postulated the theory of unbalanced growth in which destruction of part of the enzyme system throws the bacteria out of metabolic balance leading to death. Studying the effect of chlorine on bacterial enzymes, Venkobachar and coworkers (1975) observed that in the bactericidal concentration of chlorine total dehydrogenase activity in Eschericia coli was markedly affected and the inhibition correlated with percent bacteria kill. They also observed that succinyl dehydrogenase activity in the bacteria was inactivated probably due to the oxidation of sulfhydryl groups at lower chlorine doses.

Using radiolabelled chlorine, Friberg (1956) observed that the first contact oxidation reactions of chlorine with bacterial cells was responsible for bacterial death and not through chloramine formation. He later demonstrated that chlorine, even in minute amounts, reacts with and results in

destructive permeability changes in bacterial cell walls resulting in death (Friberg, 1957). Additional mechanisms of chlorine disinfection have been reported to include induction of chromosomal aberrations (Ingols, 1958); disruption of protein synthesis (Bernade et al., 1967); reaction with purines and pyrimidines of nucleic acids (Hoyano et al., 1973); oxidative decarboxylation of amino acids (Pereira et al., 1973); loss of macromolecules due to inhibition of oxygen uptake and oxidative decarboxylation (Venkobachar et al., 1975); production of lesions in DNA (Shih and Lederberg, 1976); inhibition of respiration and active nutrient transport (Camper and McFeters, 1979); disruption of bacterial spore coats and increasing spore permeability (Foegeding, 1983). Chlorine has also been reported to inactivate poliovirus by reacting with the outer protein coat of the virus (Tenno et al., 1980) or by damaging the internal RNA (Olivieri et al., 1980; O'Brien and Newman, 1979).

Of the active chlorine species, hypochlorous acid is by far the most active disinfectant. Its germicidal efficiency is related to the ease with which it can penetrate cell walls. The ease of cell penetration is comparable to that of water which is structurally similar. Also its relatively low molecular weight and electrical neutrality allow easy cell penetration (White, 1972). The germicidal efficiency of free available chlorine depends on other factors such as pH, temperature, chlorine concentration, length of contact time,

and type and concentration of microorganisms. The pH of the solution is perhaps the most important of all the above factors because an increase in pH causes a substantial decrease in biocidal efficiency and vice versa. This is because at higher pH, hypochlorous acid is converted to hypochlorite ion (equation 2) which is an inferior biocide. The hypochlorite ion obtained from hypochlorous acid dissociation is a relatively poor disinfectant because of its inability to diffuse through microbial cell walls. The inability to traverse microbial cell walls is due to the negative charge associated with it (White, 1972). Combined available chlorine as chloramines are also less effective bactericidal agents than chlorine. Chloramines are slow-acting and require more than a hundred-fold increase in contact time and much higher concentrations to achieve the same bacterial lethality as chlorine (Kabler, 1953; Butterfield, 1948).

Chemistry of Chlorine Dioxide

Chlorine dioxide is a polar gas that readily dissolves in but does not react with water to produce aqueous chlorine dioxide. It is a true dissolved gas because it does not react with water (Masschelein, 1979). The basic reaction of Davy, who discovered the gas by pouring a strong acid over sodium chlorate in 1911, is still used today (White, 1972):

$$NaClO_3 + 2HCl \longrightarrow ClO_2 + NaCl + H_2O + Cl \qquad 17.$$

Chlorine dioxide has an intense greenish yellow color with a distinctive odor similar to chlorine but more irritating and more toxic (White, 1972). One important physical property of chlorine dioxide is that it is five times more soluble in water than chlorine. It is extremely volatile and decays by photodecomposition, volatilization and decay reactions. In fact, it can easily be removed from aqueous solutions merely by aeration. It is very unstable and explosive and as such, cannot be transported but is usually prepared on site (White, 1972).

Chlorine dioxide does not belong to the family of "available chlorine" compounds, i.e., those compounds that hydrolyze to form hypochlorous acid, but due to its oxidizing power it is referred to as having an available chlorine content of 263 percent. It has a larger oxidation capacity than HOCl because it can accept 2.5 times more electrons than HOCl (Ingols and Ridenour, 1948). This can be deduced from reactions involving iodine liberation from iodide in the iodometric titration method:

$$ClO_2 + 5I^- + 4H^+ \longrightarrow Cl^- + 2.5I_2 + 2H_2O \qquad 18.$$

$$HOCl + 2I^- \longrightarrow Cl^- + I_2 + OH^- \qquad 19.$$

The oxidation potential of chlorine dioxide is less than that of HOCl as indicated by its redox potential of 0.95V compared to that of HOCl in equation 4. The redox potential of chlorine dioxide and chlorite are presented below (Masschelein, 1979):

$$ClO_2 + e^- \longrightarrow ClO_2^- \quad (0.95V) \qquad 20.$$

$$ClO_2^- + 2H_2O + 4e^- \longrightarrow Cl^- + 4OH^- \quad (0.78V) \qquad 21.$$

The oxidizing capacity of chlorine dioxide is not all used in water treatment because in practice chlorine dioxide is reduced to chlorite according to equation 20. The chlorite ion is an effective oxidizing agent and is consumed at a much slower rate in oxidation-reduction reactions.

Reaction with inorganics

In the treatment of drinking water, chlorine dioxide is commonly used for the removal of divalent ferrous iron and manganese and sometimes sulfide through oxidation reactions (White, 1972). Much higher temperatures are required for oxidation of trivalent chromium salts to chromate and will not be discussed here (Masschelein, 1979).

Oxidation of iron. In water treatment, an effective means of iron removal is through chlorine dioxide oxidation of iron from the moderately soluble ferrous (II) to the ferric (III) state as an insoluble ferric hydroxide precipitate which can be removed by filtration. The above reaction is presented in equation 22.

$$ClO_2 + FeO + NaOH + H_2O \longrightarrow Fe(OH)_3 + NaClO_2 \qquad 22.$$

The pH optimum for the above reaction is above 7.0, and is preferable in the range between pH 8-9. The reaction is a slow one, and in most cases, chlorine is the chemical of choice for this type of reaction. This is because chlorine is cheaper and equally effective as chlorine dioxide (White,

1972). Chlorine dioxide has been shown to oxidize organically bound iron (Masschelein, 1979).

Oxidation of manganese. Manganese is present in many wells and surface waters and can cause blackness of water at high concentrations. Even though it is not known to cause any health problems, the maximum level permitted in water is 0.05 mg/L (Knocke et al., 1987). Chlorine is used for manganese removal but the reaction is too slow to be desirable. Chlorine dioxide reacts more rapidly with manganese compounds than chlorine. In this reaction, manganese (II) is oxidized to manganese (IV) as insoluble manganese dioxide according to the following reaction (White, 1972):

$$2ClO_2 + MnSO_4 + 4NaOH \longrightarrow MnO_2 + 2NaClO_2 + Na_2SO_4 + 2H_2O \qquad 23.$$

The stoichiometric dosage of chlorine dioxide required for manganese oxidation has been established as 2.45 mg/mg Mn^{2+} assuming the reduction byproduct is chloride. However, under laboratory conditions, much higher doses of chlorine dioxide are required. Knocke and coworkers (1987) have recently shown that at least twice the stoichiometric amount of chlorine dioxide was required to lower manganese levels to less than 0.05 mg/L. Also, when the total organic carbon content of the water was high, much more chlorine dioxide was required than permitted by law to limit manganese content to acceptable levels.

Optimum conditions for the reaction have not been fully elucidated but it is thought that complete oxidation of manganese occurs at pH values higher than 7 (White, 1972). Like iron, chlorine dioxide will oxidize organically bound manganese. The chlorite formed from chlorine dioxide reduction can also be used to reduce manganese or iron under similar conditions (Masschelein, 1979).

Oxidation of sulfides. Hydrogen sulfide is found dissolved in underground waters and has been shown to react with chlorine to produce sulfates and elemental sulfur. Chlorine dioxide oxidation of hydrogen sulfide to sulfate occurs extensively in the pH range 5-9 (White, 1972). The type of products formed, either elemental sulfur or sulfate, is dependent on pH and temperature, with higher temperatures and pH favoring sulfate formation (Dohnalek and FitzPatrick, 1983). Reactions of chlorine with sulfur has fully been investigated and characterized but the same does not hold true for chlorine dioxide. Until more data becomes available on chlorine dioxide oxidation of sulfides, chlorine remains the chemical of choice for sulfide removal since it is cheaper and data is available on the type of byproducts likely to be encountered.

Reaction with organics

Chlorine dioxide is a selective oxidant which reacts with organic matter via oxidation to produce both volatile and non-volatile reaction products. In some cases, however, some

chlorinated compounds are encountered. This type of chlorination reaction is thought to be an indirect one and probably corresponds to a progressive reduction of the dioxide passing through the hypochlorous acid stage (Masschelein, 1979). These chlorination reactions involving chlorine dioxide arise presumably due to the presence of chlorine as a contaminant in the dioxide used (Masschelein, 1979). Chlorine dioxide does not react with ammonia to form chloramines; therefore, chloramine toxicity is avoided with its use. Unsubstituted aromatics, primary amines and many other organic compounds are unreactive towards chlorine dioxide (Raugh, 1979).

Oxidation of phenols and odor control. Phenols are ubiquitous pollutants in water and the environment and have been used as model compounds for chlorine reactions and haloform formation (Aieta and Berg, 1986). Oxidation of phenols and phenolic compounds by chlorine and chlorine dioxide has been extensively investigated because phenolic tastes and odors are produced in natural waters as a result of industrial waste pollution of raw water. Petroleum and wood processing plants, algae and decaying vegetation are principal sources of phenolic compounds. Chlorination of polluted waters leads to the formation of chlorophenols such as 2-chlorophenol , 2,4-dichlorophenol, 2,4,6-trichlorophenol and others which possess distinct offensive tastes and odors (White, 1972).

Chlorine dioxide has been shown to be specific for the control of these phenolic and chlorophenolic tastes and odors many times faster than free available chlorine. This is related to the high oxidizing capacity of chlorine dioxide when compared to chlorine (Masschelein, 1979; White, 1972). Apart from control of phenolic tastes and odors, chlorine dioxide has successfully been used against algae odors, fishy tastes, and earthy-musty taste and odor compounds (Lalezary et al., 1986; Wright, 1980; Medker et al., 1968).

Reaction of equal parts of chlorine dioxide with phenols has been reported to yield p-benzoquinone and 2-chloro-benzoquinone in the concentration range 1-1000 mg phenol/L. Organic acids, mainly maleate and oxalate are also formed if the ratio of chlorine dioxide to phenol is increased five-fold (Wajon et al., 1982; Masschelein, 1979). Extended oxidation with excess chlorine dioxide leads to the formation of carboxylic acids and, under moderate conditions, quinones. When excess phenols are used, chlorophenols are formed due to the slow release of hypochlorous acid which subsequently reacts with excess phenol (Wajon et al., 1982). Under water treatment conditions, the chlorine dioxide to phenol ratio will be greater than unity and the major products likely to be encountered are p-benzoquinone and simple organic acids.

Other oxidative reactions. Carbohydrates, proteins and amino acids and other food components have been shown to undergo mainly oxidative reactions with chlorine dioxide and are

discussed later in this chapter. Environmentally significant compounds such as polynuclear aromatic hydrocarbons (PAHs) and their heterocylcic analogs also react with chlorine dioxide to varying degrees. Lin and Carlson (1984) subjected some environmental heterocycles to dilute aqueous solutions of chlorine, chloramine and chlorine dioxide and the latter was found to produce mainly oxygenated byproducts. For example, chlorine dioxide oxidation of indole produced oxindole and isatin.

Chlorination reactions. As mentioned earlier, chlorination or chlorine incorporation is not an intrinsic reaction of chlorine dioxide. These reactions are secondary reactions which are thought to occur through the release of hypochlorous acid from a primary product or from impurities in the chlorine dioxide (Masschelein, 1979). In reactions involving chlorine and chlorine dioxide incorporation into lipids, Ghanbari et al. (1982b) showed that chlorine incorporation was many orders of magnitude higher with chlorine than chlorine dioxide.

Chlorine dioxide disinfection

Even though the bactericidal properties of chlorine dioxide were known at the beginning of this century, the practical uses of said properties were not fully utilized until the middle of the century. This is because chlorine dioxide was first applied to water treatment around the middle of the century and also increased environmental and water pollution led to a search for alternate and varied

disinfectants (Masschelein, 1979; White, 1972). McCarthy (1944) first recommended the use of chlorine dioxide for the disinfection of water of low organic content. This is because increasing the organic content of water decreased the germicidal effectiveness of chlorine dioxide. Ridenour and Ingols (1947) showed that chlorine dioxide was as effective as chlorine for the control of E. coli and other bacterial populations. These workers were the first to show that unlike chlorine, the bactericidal efficiency of chlorine dioxide was unaffected over a wide pH range (from pH 6-10), which makes it suitable for the disinfection of high pH waters. On the other hand, the germicidal effectiveness of chlorine dioxide was found to decrease at low temperatures (Ridenour and Ambruster, 1949).

Most of the studies regarding chlorine dioxide disinfection have focussed on a comparison with that of chlorine and, as a result, very little information is available on its mode of action. Some of the comparative studies have shown that chlorine dioxide is a more efficient virucide and bactericide than chlorine (Alvarez and O'Brien, 1982; Aieta et al., 1980; Longley et al., 1980; Sawyer, 1976). The proposed mechanisms of action of chlorine dioxide on microbes are similar to those of chlorine; either through a reaction with macromolecules or through gross physiological changes. Like chlorine, there is evidence that chlorine dioxide acts on dehydrogenase enzymes located in bacterial

cytoplasmic membranes but this evidence has not been substantiated.

Chlorine dioxide is reactive towards several compounds including phenols, some amino acids and proteins and this reactivity has been used to explain its bactericidal action. Fujii and Ukita (1957) demonstrated that chlorine dioxide oxidation of tryptophan produced some indole derivatives of tryptophan which may explain the bactericidal action of chlorine dioxide. On the mode of killing action of chlorine dioxide on bacteria, Bernade and coworkers (1967) concluded that chlorine dioxide was sufficiently reactive with several amino acids to alter their structures and thus, react with cell walls. They also observed that protein synthesis was abruptly inhibited by the action of chlorine dioxide. Roller et al. (1980) have reported that under laboratory conditions, the level of chlorine dioxide used in treatment plants does not affect DNA or protein synthesis. Contrary to the above, Alvarez and O'Brien (1982) in their studies on poliovirus concluded that chlorine dioxide reacted with viral RNA leading to virus inactivation.

Noss and others (1983) investigated the reactivity of chlorine dioxide with nucleic acids, proteins and amino acids. Chlorine dioxide readily and rapidly reacted with the amino acids cysteine, tyrosine and tryptophan but not with viral RNA. The authors postulated that the virucidal activity of chlorine dioxide was probably due to alteration of viral

proteins. Further reports by the same authors suggest that chlorine dioxide reaction with tyrosine appears to be the main mechanism of action of F2 virus inactivation by chlorine dioxide (Olivieri et al., 1985).

Another probable mechanism of action of chlorine dioxide may be due to its interaction with membrane lipids and fatty acids leading to permeability changes in membranes. Ghanbari et al. (1982b) showed that chlorine dioxide was highly reactive towards free fatty acids leading to the formation of mainly oxidized byproducts. The authors observed that free fatty acids were more reactive with chlorine dioxide than their methyl esters. From the foregoing discussion, it is possible that chlorine dioxide oxidation of membrane lipids and proteins leads to microbial death. Even though some of the reactions have been reported to occur in model systems, the actual mechanisms that take place in the intact microorganisms leading to death still need to be proven.

Reactions of Aqueous Chlorine with Amino Acids, Peptides and Proteins

Proteins are complex high molecular weight constituents of plant and animal matter made up of carbon, nitrogen, oxygen, hydrogen and, in some cases, sulfur. Of these elements, carbon, nitrogen and sulfur exert a chlorine demand (White, 1972). Proteins and protein material from many sources contribute greatly to the organic nitrogen content of

natural waters and pose a problem during water chlorination (Qualls and Johnson, 1985). Amino acids are water soluble and are typically found in natural waters at concentrations in the range 2-400 μg/L (Le Cloirec and Martin, 1985). During water treatment, the amount of amino acids can increase substantially due to hydrolysis-depolymerization of proteins or peptides, or due to microbial metabolism (Le Cloirec and Martin, 1985). In the food industry, amino acid concentrations are likely to be much higher in process cleaning waters, and superchlorination of process waters is sometimes used to control microbial contamination (Robinson et al., 1981).

The reactions of chlorine with amino acids, proteins and peptides have been studied in detail and reported to produce both oxidized and chlorinated byproducts under different treatment conditions (Jolley et al., 1978; Pereira et al., 1973). The first report in the literature on the chlorination of amino acids was made by Langheld (1909) who reported that sodium hypochlorite reacted with alpha-amino acids to form mono- or dichloroamino acids which subsequently decomposed to form aldehydes. Langheld (1909) observed that the rate of monochloramine decomposition was affected by the amino group; primary amino groups decomposed to give aldehydes or ketones as well as ammonia while secondary amino groups produced amines instead of ammonia. Dakin et al. (1916) and Dakin (1916) confirmed Langheld's study and showed that nitriles

were formed as major products when excess chlorine was used. Dakin and colleagues showed that hydrogen cyanide was produced from glycine, acetonitrile from alanine, isobutyl cyanide from leucine and cyanobenzene from phenyl acetate. The general mechanism proposed by Dakin (1916) involves the initial formation of a dichloroamino acid which undergoes further decomposition to form a cyanide. The chlorination of several amino acids including those studied by Dakin and coworkers has been further investigated (Isaac and Morris, 1983a; Margerum et al., 1979). These studies showed that at neutral pH all the amino acids tested readily reacted with chlorine with the possible involvement of N-chloro compounds as intermediates. Rydon and Smith (1952) developed a paper chromatographic method for determining N-chloroamino acid formation during chlorination. They showed that several amino acids including glycine, alanine, leucine, serine, threonine, lysine, phenylalanine and histidine liberated chlorine from the corresponding N-chloro derivatives which reacted positively with a starch-potassium iodide spray. Cysteine, cystine, methionine and tyrosine failed to release chlorine due probably to the formation of mainly oxidized products.

The importance of pH in product formation was investigated by Wright (1936) who observed that aldehydes were formed from alpha-amino acids on reaction with sodium hypochlorite at neutral pH while nitriles predominated at lower pH. From his point of view, acidity favors chlorination

and alkalinity favors oxidation. Wright showed that under acidic conditions glycine was first chlorinated, and the resulting chloroamino acid was then oxidized by any available chlorine present; whilst under alkaline conditions, destruction of available chlorine was mainly due to oxidation. Jolley and Carpenter (1983) also observed that at neutral pH, monochlorinated amino acids were formed with aqueous chlorine, while dichloro derivatives were produced as major products under acidic conditions. Stanbro and Smith (1979) studied the kinetics and mechanisms of decomposition of N-chloroalanine in aqueous solution and showed that the decomposition products included acetaldehyde, ammonia and carbon dioxide and, depending on pH, pyruvate. Le Cloirec and Martin (1985) have provided additional evidence to support the general mechanisms already proposed. A summary of the general reaction mechanisms leading to the formation of nitriles and aldehydes (Figure 4) has been provided by Le Cloirec and Martin (1985).

In the reactions leading to the formation of aldehydes and nitriles, imine-type compounds are known as essential intermediates but have not been isolated in the chlorination of individual amino acids. However, in the case of a peptide, DL-alanyl-DL-leucine, an imine-type compound has been isolated and characterized (Pereira et al., 1973). Pereira et al. (1973) also showed that nitriles are major and aldehydes are minor products of amino acid chlorination reactions. Tan et al. (1987a) studied the kinetics of the reactions of aqueous

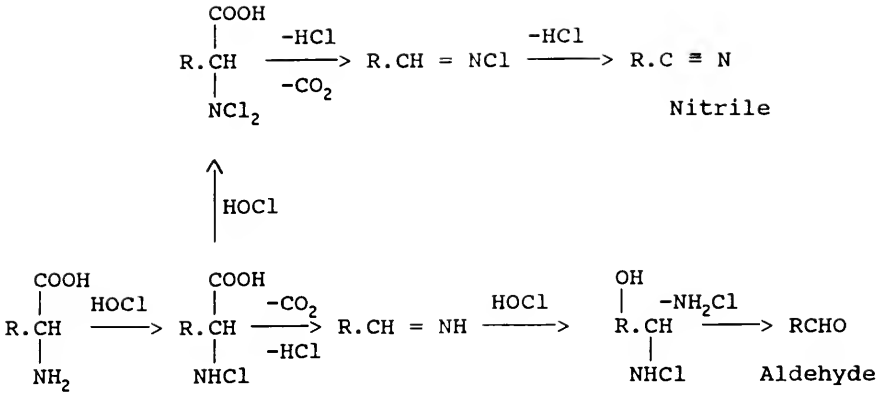

Figure 4. Reactions of chlorine with amino acids leading to the formation of nitriles and aldehydes (Adapted from Le Cloirec and Martin, 1985)

chlorine and several amino acids in different buffers and observed that all the amino acids were reactive towards chlorine over a wide pH range. The type of buffer system was found markedly to affect the reaction rates. These workers did not identify any of the reaction products but confirmed previous reports (Isaac and Morris, 1983a) that the reaction rates of the sulfur-containing amino acids, cysteine and methionine, as well as tryptophan were too rapid to be monitored by the iodometric method of titration. Jancangelo and Olivieri (1985) have also reported that the sulfur-containing amino acids and the heterocyclic amino acid, tryptophan, are the most reactive with monochloramine.

The specific functional groups of the amino acids have been shown to affect the type of products likely to be formed during chlorination. In the case of the sulfur amino acid, cysteine, Pereira and colleagues (1973) have shown that the major products of chlorination were cystine and cysteic acid while cystine produced only cysteic acid as the end product. Amino acids with phenyl or heterocyclic groups readily form chlorinated analogs if the rings are sufficiently activated. For example tyrosine and tryptophan have been shown to form the corresponding mono- and dichloro derivatives upon aqueous chlorination (Trehy et al., 1986; Burleson et al., 1980; Pereira et al., 1973). Aqueous chlorination of tyrosine has been reported to yield mono- and dichlorobenzeneacetonitrile and mono- and dichlorobenzeneacetaldehyde (Trehy et al., 1986;

Burleson et al., 1980; Pereira et al., 1973). Major chlorination products of phenylalanine were previously reported to be phenylacetonitrile (95%) and phenylacetaldehyde (5%) but some chlorinated derivatives have been identified as well (Horth et al., 1987).

In the case of tryptophan, Kirk and Mitchell (1980) observed the formation of a dark-colored precipitate when reacted with different concentrations of aqueous chlorine. Although the reaction products were not fully identified, the authors isolated and partially characterized a monochlorinated product. Using structurally similar indole compounds, they showed that the intact heterocyclic ring was very important in color formation. Such color changes have been used colorimetrically to determine chlorine dioxide concentrations in water upon reaction with tyrosine (Hodgen and Ingols, 1954). Tryptophan has also been reported to produce oxidized indole derivatives as well as ether extractable chlorinated analogs (Owusu-Yaw et al., 1989; Trehy et al., 1986). Nitriles and aldehydes are also produced as a result of aqueous chlorination of tryptophan (Owusu-Yaw et al., 1989; Horth et al., 1987).

The products of the reactions of chlorine with peptides and proteins have been reported to be mainly N-chloro or N,N-dichloro derivatives (Pereira et al., 1973, Rydon and Smith, 1952). Rydon and Smith (1952) showed that peptides and proteins reacted with chlorine gas to form

N-chloropeptides which could be detected visually on paper chromatograms using a starch-potassium iodide spray. According to the authors, color was developed from the liberation of chlorine from the N-chloropeptide bond which reacted with the starch iodide-indicator. Pereira and coworkers (1973) investigated the reactions of excess chlorine with the four dipeptides, glycyl-L-alanine, glycyl-DL-phenylalanine, DL-alanine-DL-leucine and L-valyl-L-valine and showed that the corresponding N,N-dichloro-dipeptides were the major reaction products. From their point of view, the amide nitrogen of the peptide was not attacked, and the peptide bond itself was relatively resistant to chlorination. These authors were the first to show that N-chloroimines were produced by the dehydrochlorination of the N,N-dichloro-dipeptide DL-alanyl-DL-leucine with ethanolic sodium hydroxide at ambient temperature.

Glycylglycine has been used as a model for the formation of N,N-dichloroglycylglycine and N-chloroglycylglycine (Isaac and Morris, 1985; Qualls and Johnson, 1985; Margerum et al., 1979). These studies show that the rate of chlorination of the dipeptide is faster than that of several amino acids and ammonia and that the peptide will be preferentially chlorinated over ammonia or the amino acids glycine, alanine, serine and glutamate in water. Other peptides such as phenylalanylglycine and glycylglycylalanine have been reported to produce stable chloramine residuals (Horth et al., 1989).

From all the studies cited, it becomes clear that the fastest initial reactions of peptides with chlorine occur at the nucleophilic terminal amino groups or the reactive side groups of amino acids. For example when flour was chlorinated with excess chlorine gas, the levels of the amino acids, methionine, threonine, cysteine and histidine in the proteins decreased by approximately, 68, 41, 32 and 17%, respectively (Ewart, 1968).

Tan et al. (1987a) studied the kinetics of the reactions of aqueous chlorine and three peptides and observed that the rates of reaction of L-glycyl-L-tryptophan and L-tryptophyl-glycine were too rapid to be measured iodometrically. The authors suggested that the rapid reaction rates were due mainly to the reaction of chlorine with the heterocyclic ring of tryptophan and not with the amino or carboxyl groups. In the case of aspartame, even though the component amino acids, aspartate and phenylalanine, were individually reactive towards chlorine, the dipeptide was not. The authors did not provide any conclusive evidence as to whether any of the reaction products were chloramines. Apart from the formation of N-chloro compounds, Tsen and Kulp (1971) have postulated that amino acid side chains of proteins are progressively cleaved by increasing concentrations of chlorine. They observed that the amount of water-extractable proteins in chlorinated flour increased as the chlorine concentration was increased. Chlorine has also been shown to be readily

incorporated into proteins from different sources including shrimp, meat and poultry (Johnston et al, 1983; Cunningham and Lawrence, 1977); egg albumin, gelatin, casein, and bovine serum albumin (Baker, 1947; Wright, 1936 and 1926; Milroy, 1916); and flour proteins (Wei et al., 1984).

Reactions of Chlorine Dioxide with Amino Acids, Peptides and Proteins

Chlorine dioxide has been proposed as an alternate to chlorine for disinfection purposes and it is therefore important to understand the kind of reactions and byproducts likely to be formed with its use, especially proteinaceous material. The reactions of chlorine dioxide under water treatment conditions are mainly oxidative and as such, it has been used for the control of phenolic tastes and odors in natural waters (White, 1972). Several reports are available in the literature on the reactions of chlorine dioxide with amino acids under different conditions, but as pointed by Masschelein (1979), the conditions of the reaction are very important.

In earlier investigations, Schmidt and Braunsdorf (1922) observed that most amino acids were not reactive towards chlorine dioxide and that the least reactive were glycine, leucine, serine, alanine, phenylalanine, glutamate, asparagine, valine and hydroxyproline. The authors found cystine to be very reactive. Bernade et al. (1967) further

investigated the reaction of chlorine dioxide with several amino acids and observed that after a contact time of 30 minutes, asparagine, histidine, phenylalanine, arginine, proline and leucine were unreactive. Contrary to the above findings, Kennaugh (1957) noted that when a mixture containing tryptophan, tyrosine, proline, threonine, methionine, valine and lysine were mixed with diaphanol, a solution of 50% acetic acid saturated with chlorine dioxide, the aromatic amino acids disappeared after 18 hours. Also, most of the amino acids were found to react after 3 days and phenylalanine did not produce any ninhydrin positive material. Noss et al. (1983) studied the reactivity of equimolar concentrations (2.5×10^{-4} M) of twenty-two amino acids and four nucleic acids with chlorine dioxide at neutral pH and found only seven (cysteine, cystine, proline, histidine, hydroxyproline, tyrosine and tryptophan) to be reactive. Additional evidence in support of these studies was provided by Tan et al. (1987b) who showed that six of twenty-one amino acids were reactive with chlorine dioxide. Of the six, the reactions of cysteine, tryptophan and tyrosine were too rapid to be measured by the iodometric titration method.

From the above and other studies, it is clear that the reactivity of chlorine dioxide with amino acids and proteins depends on pH, temperature and contact time, with higher temperatures and contact times and lower pH favoring the reaction. Also, specific functional groups of the amino acids

and proteins such as phenyl or sulfhydryl and the relative molar concentrations of reagent and substrate affect the reactions. For example, glycine and phenylalanine were inert toward chlorine dioxide at room temperature when equimolar concentrations of reagent and substrate were used (Noss et al., 1983). However, the reactions were found to proceed when excess molar concentrations of chlorine dioxide were used (Taymaz et al., 1979).

The sulfur-containing amino acids and those with phenyl or heterocyclic rings have been reported to be the most reactive with chlorine dioxide (Masschelein, 1979). Of the amino acids containing sulfur, cystine has been reported to produce cysteic acid with a cystine disulfoxide intermediate. The reaction is said to involve the breakage of the sulfur-sulfur bond of cystine followed by further oxidation (Masschelein, 1979). Under similar conditions, methionine is oxidized to the sulfoxide and then to the sulfone. Other organic sulfides and mercaptans are also known to undergo similar oxidative reactions with chlorine dioxide.

Chlorine dioxide oxidation of tyrosine is similar to those of phenols which are especially susceptible to oxidation. When pure chlorine dioxide is used, no chlorinated products are obtained (Masschelein, 1979). With chlorine dioxide, tyrosine has been reported to produce pink dopaquinone and red dopachrome. This reaction has been used as a basis for the colorimetric determination of chlorine

dioxide concentrations in water (Hodgen and Ingols, 1954). When excess chlorine dioxide, 3 moles per mole of tyrosine, is used for an extended period of time, other unidentified colored products are formed as well. Reaction of 2 molars excess of chlorine dioxide with phenylalanine at elevated temperatures has been reported to produce phenylacetic acid, phenylacetaldehyde, benzoic acid, benzaldehyde and mandelic acid (Taymaz et al., 1979). No chlorinated products were identified from the above reaction. When the chlorine dioxide concentration was increased to 10 excess molar under similar conditions, no phenylalanine or above-mentioned products were detected after 6 days. From the foregoing, it is possible that further oxidation of the initial reaction products takes place with excess chlorine dioxide thus giving off carbon dioxide and causing the disappearance of substrate.

The heterocyclic group of compounds is also very susceptible to chlorine dioxide oxidation leading to the formation of complex oxidized products. Chlorine dioxide oxidation of tryptophan produced indoxyl, isatin, indigo red as well as unidentified polymerization products (Fujii and Ukita, 1957). Structurally similar indole derivatives such as indole itself, 3-methyltryptophan, 3-indolelactic acid and 3-methylindole have also been shown to be readily oxidized by chlorine dioxide, sometimes leading to color and precipitate formation (Sen et al., 1989; Lin and Carlson, 1984). Indole is oxidized to oxindole and isatin while 3-methylindole

produces 3-methyloxindole and o-formamidoacetophenone (Lin and Carlson, 1984).

Unlike amino acids, the reaction of chlorine dioxide with proteins and peptides has not received much attention in model systems. A review of the available literature has been documented by Fukayama et al. (1986). Tan et al. (1987b) studied the kinetics and mutagenicity of three peptides with chlorine dioxide at room temperature using equimolar concentrations of reagent and substrate. They observed that the reactions of two dipeptides L-tryptophyl-glycine and L-glycyl-L-tryptophan were too fast to be determined by iodometric titration. They attributed the very fast reaction rates to the presence of the tryptophan moieties which have previously been shown to react rapidly with chlorine dioxide (Fujii and Ukita, 1957). The third dipeptide, aspartame, was found to be relatively unreactive since less than 10% of the initial reagent was lost after 2 hours of reaction. The authors explained that the inertness of this dipeptide with chlorine dioxide could be attributed to the component amino acids, aspartate and phenylalanine, which were individually unreactive towards chlorine dioxide.

Chlorine gas and chlorine dioxide have traditionally been used to treat flour to improve baking properties and dough quality. Meredith et al. (1956) treated wheat flour with high levels of chlorine dioxide and did not find any chlorinated derivatives of amino acids and proteins. They, however, found

that the levels of the amino acids, cysteine and tryptophan were decreased by 25 and 8%, respectively. Moran and coworkers (1953) reported that methionine was oxidized to the corresponding sulfoxides and sulfones and cysteine to cysteic acid in flour treated with chlorine dioxide. Ewart (1968) exposed solutions of wheat proteins to chlorine dioxide and observed the formation of a pink-yellow color due to the oxidation of tryptophan and tyrosine. Under similar conditions there was an increase in chlorine dioxide-induced solubility of flour proteins. In the treatment of wool, chlorine dioxide has been reported to cause a dissolution of up to 40% of the proteinaceous matter leading to losses in weight. These weight losses have been attributed to the reactivity of amino acid components of the wool with chlorine dioxide (Masschelein, 1979). Tyrosine, methionine, glutamate and tryptophan were the most reactive. The reactivity of tryptophan with chlorine dioxide was found to be greater than that of tyrosine and usually led to the formation of a reddish coloration (Masschelein, 1979).

Toxicological Assessment

Evidence already presented suggests that both chlorine and chlorine dioxide have the capacity to react with a wide range of organic compounds under different treatment conditions. Many organic substances in food and potable water are capable of reacting with chlorine to produce both

halogenated and oxidized products. Chlorine dioxide on the other hand produces mainly oxidized reaction products. Even the most gentle chlorination treatments are likely to produce chlorinated and non-chlorinated organics. Chlorination of organic compounds has been shown to increase their lipophilic nature and thereby increase their toxicity and/or their ability to bioaccumulate (Kopperman et al., 1978). This section will focus on the toxicology of chlorine and compounds arising from the use of chlorine and chlorine dioxide disinfection and their possible health hazards.

Overview

Chlorine has been used as the major disinfectant for water supplies for many years and has been a major factor in reducing the transmission and spread of numerous waterborne diseases and infections. Concerns about the use of chlorine for water treatment came to light when it was discovered that THMs are produced as a result of water chlorination practices (Bellar et al., 1974; Rook, 1974). These concerns gained additional impetus when the National Cancer Institute reported that chloroform, a major byproduct of chlorination, is carcinogenic in two laboratory animals (NCI, 1976). Because of this discovery, the United States Environmental Protection Agency amended the National Interim Primary Drinking Regulation which set up guidelines for the control of THM concentrations in drinking water. A maximum contaminant level of 0.10 mg/L was set for total THMs in finished drinking water

in the United States (Symons et al., 1981). In order to comply with these guidelines, several researchers in the area have devoted their efforts to the search for alternate disinfectants which will produce very little or no THM in finished drinking water (Condie, 1986; Lykins and Griese, 1986; Symons et al., 1978).

Chlorine dioxide was selected as the alternate oxidant and disinfectant for drinking water because in most aspects it is similar or superior to chlorine and it produced very little or no THM (Knocke et al., 1987; Aieta and Berg, 1986; Lykins and Griese, 1986; Lauer et al., 1986; Aieta et al., 1980). Also, it has been in use for many years in several European countries without any reported adverse effects (Symons et al., 1978). According to Symons and coworkers, a 1977 survey showed that about 103 facilities were using or had used chlorine dioxide in the United States. The suggested use of chlorine dioxide was welcomed by several researchers and numerous reviews are available regarding its action, especially for THM control. One of the main disadvantages of chlorine dioxide is its additional cost to the consumer which is estimated at about three times that of chlorine. However, a report by Lykins and Griese (1986) in Evansville, Indiana, shows that apart from being suitable for THM control, chlorine dioxide is also cost effective (about $1.77/resident/year). On the other hand, it becomes clear that the toxicological problems and possible health hazards associated with chlorine

dioxide and its disinfection byproducts are not immediately known (Condie, 1986).

Unfortunately, THMs are not the only byproducts of disinfection which pose possible health hazards in drinking water. Other chlorinated and non-chlorinated organic compounds, both volatile and non-volatile, have been reported to be produced during water chlorination (Loper et al., 1985; Coleman et al., 1983; Cheh et al., 1980; Christman, 1980). Organic compounds that have been identified in drinking water are mostly volatile and represent only about 5-10% of the total organic compounds in drinking water (NAS, 1977). Of the 250 or more compounds previously identified in drinking water, about 10% have been classified as being carcinogenic and a good number remain to be assessed for genotoxicity (Kraybill, 1978). According to the latest survey, more than 1,100 organic compounds have been identified in drinking water, most of which are at or below the μg/L level (Lucas, 1985).

Another group of compounds, haloacetonitriles (HANs), has been identified in drinking water which has compounded the problem (Daniel et al., 1986; Bierber and Trehy, 1983; Oliver, 1983; Trehy and Bierber, 1981). Proteinaceous material, especially amino acids, as well as fulvic and humic substances have been implicated and confirmed precursors of both THMs and HANs upon reaction with chlorine (Bierber and Trehy, 1983; Oliver, 1983; Rook, 1980). A third class of compounds which has received very little attention is the

relatively high molecular weight compounds which are not easily amenable to gas chromatographic analysis. This class of compounds is mostly non-volatile and several have been shown to be mutagenic in short-term assays (Cheh et al., 1980). The difficulty encountered in their isolation and identification is that they are mostly non-volatile and difficult to identify using current analytical techniques (Meier et al., 1983). Some are polymerization products produced from the condensation of several individual compounds which makes identification more difficult even with up-to-date instrumentation. Due to these analytical limitations, the toxicology and possible health risks posed by this class of compounds still remain unknown.

Toxicology of chlorine and chlorine dioxide

Chlorine forms hypochlorous acid (HOCl) which dissociates to form hypochlorite ion (OCl^-) in solution. Under alkaline conditions, chlorine dioxide disproportionates to form chlorite (ClO_2^-) and chlorate (ClO_3^-). Most of the current literature on the toxicology of chlorine dioxide and chlorine deals with the undesirable byproducts arising from the reactions of these disinfectants with organic materials in water. As a result, very little information is available on the toxicology of the disinfectants themselves or the endproducts of their decomposition such as chlorite, and hypochlorite. This section reviews the available work concerning the toxicology of chlorine and chlorine dioxide,

as well as their decomposition byproducts, hypochlorite, chlorate and chlorite.

Chlorine, hypochlorous acid and hypochlorite

Information dealing with the toxicity of chlorine is somewhat conflicting. Blabaum and Nichols (1956) reported that no gross abnormalities occurred in mice fed chlorinated drinking water for 30 or 50 days. Also, a seven generation study in rats administered chlorine in their drinking water did not result in any observable adverse toxic or teratogenic effects. Other reports on the toxicity of chlorine or chlorinated water indicate that toxicity, if any, is not due to chlorine itself but probably due to its reaction with precursors in the stomach. Chang et al. (1981) have reported that administration of chlorinated water led to the development of fatty liver in rats with a better than two-fold increase in liver triglycerides. The acyl groups of triacylglycerols and phospholipids were also affected. Such effects were completely reversible when dosing was discontinued and the animals recovered fully after 10 days. The induction of fatty liver may be due to the formation of chloroform with precursor substances in the body since chloroform has been shown to give rise to this condition (Cornish, 1980).

No toxic effects were observed in rats and guinea pigs fed up to 400 ppm of sodium hypochlorite in milk and water (Cunningham, 1980). At low dose levels, chlorine was actually

shown to act as a broad spectrum antibiotic and stimulated the growth rates of the rats and guinea pigs. However, at very high doses (2000 ppm), Cunningham observed significant increases in liver weights probably due to the formation of fatty liver described by Chang and coworkers (1981). In earlier studies, Cunningham and Lawrence (1977) fed flour, chlorinated with 2,000 and 10,000 ppm chlorine, to rats and observed decreased growth rates and increased liver and kidney weights. The authors reported similar effects in rats fed chlorinated flour lipids and proteins (Cunningham and Lawrence, 1978).

The tumor initiating and/or promoting activities of chlorine have also been studied but the results are still inconclusive. Hayatsu and coworkers (1971) observed that sodium hypochlorite acts as a cocarcinogen in mice and increased tumor yields when applied in conjunction with a sub-threshold dose of 4-nitroquinoline-1-oxide. Contrary to these observations, Pfeiffer (1978) reported that sodium hypochlorite decreased the carcinogenic activity of benzo(a)pyrene in mice.

Chlorine has been reported to induce chromosomal aberrations in mammalian cells *in vitro* (Mickey and Holden, 1971), and sodium hypochlorite, which is commonly used in water disinfection, has been shown to inhibit preferentially the growth of DNA polymerase-deficient strains of *E. coli* (Rosenkranz, 1973). These mutagenic events are due to the

reaction of chlorine with the DNA of living matter (Rosenkranz, 1973). Sodium hypochlorite and not hypochlorous acid (Thomas et al., 1987) has also been reported to be weakly mutagenic for Salmonella typhimurium in the Ames Salmonella/ microsome assay (Wlodkowski and Rosenkranz, 1975). It resulted in a base-pair substitution mutation in Salmonella presumably due to a reaction with purine and pyrimidine bases which has previously been demonstrated to occur in vitro (Hoyano et al., 1973). Meier et al. (1983) have demonstrated that sodium hypochlorite, and not hypochlorous acid, was capable of inducing sperm-head abnormalities in mice when fed various chlorine compounds by gavage. From the evidence provided, it is not clear whether the mutagenic and/or carcinogenic effects are due to chlorine itself, or due to the action of some reaction byproducts. It has been reported that chloroform, di- and trichloroacetic acid as well as di- and trichloroacetonitrile were formed in vivo in rats following oral administration of sodium hypochlorite (Mink et al., 1983). Some of these byproducts may play a role in the observed genotoxic effects and will be discussed in the latter section.

Recent studies by Revis and coworkers (1985) have shown that chlorine residuals similar to those found in drinking water increased heart rate, heart fibrous tissue and plasma cholesterol levels in rabbits and pigeons by interacting with a diet marginal in calcium. They suggested a relationship

between water chlorination and hypothyroidism which they assumed to be caused by the production of chlorinated products. In earlier studies Revis et al. (1983) also observed signs of myocardial hypertrophy and arteriosclerosis in rabbits and pigeons fed chlorine as sodium hypochlorite in their drinking water. These changes were reversed when calcium intakes in the diets were restored to normal.

Chlorine, as chloramine, has received very little attention in terms of its toxicological potential. It has been reported to be weakly mutagenic in bacteria (Shih and Lederberg, 1976) but does not produce sperm-head abnormalities in mice (Meier et al., 1985). In a very recent study, both mono- and dichloramines and their derivatives were shown to be mutagenic in the Ames assay (Thomas et al., 1987). The authors showed that S. typhimurium strain TA100 was more sensitive to chloramine mutagenesis and that the lipophilic dichloramines were more active than the corresponding monochloramines. Chloramine as the synthetic disinfectant, chloramine-T, has also been reported to cause sister chromatid exchanges in cultured Chinese hamster ovary cells in vitro (Weitberg, 1987). Chloramine has also been evaluated for teratogenicity in developing rat fetuses but showed little or no adverse effects at levels up to 100 ppm (Abdel-Rahman et al., 1982).

Chlorine dioxide, chlorate and chlorite

The toxicology of chlorine dioxide as well its inorganic byproducts, chlorate (ClO_3^-) and chlorite (ClO_2^-), has been studied extensively because of their observed effects on the hematopoietic system. All three compounds are usually evaluated together because both chlorine dioxide and chlorate can rapidly be converted to chlorite, and therefore, some aspects of their toxicology are expected to be similar (Condie, 1986). Like chlorine, considerable disagreement exists in the literature concerning the toxicity of chlorine dioxide and its reaction products. Chronic administration of chlorine dioxide and chlorate to rats in their drinking water did not produce any observed toxic effects at doses up to 10 mg/L. However, higher mortality rates were observed in rats administered 100 mg/L for 2 years (Haag, 1949).

Chlorite has been shown to be a potent stressor of blood and produces methemoglobinemia and hemolytic anemia both *in vivo* and *in vitro* (Hefferman et al., 1979a; 1979b). The dose required to produce hemolytic anemia is much lower than that required for methemoglobinemia. It has also been reported to cause hemolytic anemia, increase osmotic fragility and glucose-6-phosphate dehydrogenase activity in erythrocytes, and reduce growth and conception rates in mice (Moore and Calabrese, 1980a; 1980b; Moore et al., 1980). Further studies have established that all three compounds produce the same hemolytic effects in test animals as well as decrease

glutathione levels in rats (Abdel-Rahman et al., 1984; Abdel-Rahman et al., 1979; Couri and Abdel-Rahman, 1979). Chlorine dioxide induced reduction in glutathione levels tended to disappear with continued exposure (Couri and Abdel-Rahman, 1979). The mechanism of the hemolytic activity of chlorine dioxide and its metabolites has been attributed to the production of hydrogen peroxide which, like other strong oxidants, produces this effect (Bull, 1983).

Chlorite and chlorate, but not chlorine dioxide have been reported to produce hematological effects in nonhuman primates (Bercz et al., 1982). Chlorine dioxide, on the other hand, caused a decrease in serum thyroxin levels in monkeys. The hypothyroid effect was unique to chlorine dioxide since its endproducts, chlorite and chlorate, did not show this effect even at higher doses (Bercz et al., 1982). Since chlorine dioxide is a very reactive oxidant, it is not known whether the decrease in thyroxin levels is due solely to this disinfectant or due to some primary oxidation products formed with precursor molecules in the gastrointestinal tract. Other studies showed no significant decreases in serum thyroxin levels in adult rats fed up to 100 mg/L chlorine dioxide (Condie, 1986). However, administration of similar doses to pregnant and lactating females resulted in decreased serum thyroxin concentrations in rat pups (Condie and Bercz, 1985; Orme et al., 1985).

Reproductive and teratogenic studies have also been performed in laboratory animals. Exposure of female mice to chlorine dioxide in their drinking water from breeding to weaning resulted in reduced growth rates and body weights (Moore et al., 1980), and at much higher concentrations, increased stillbirths and fetal reabsorptions (Couri et al., 1982). Contrary to these findings, Suh and coworkers (1983) did not observe any maternal toxicity or gross changes in fetal weight, litter size or skeletal anomalies in the development of rats fed chlorine dioxide, chlorite and chlorate in their drinking water. Further studies did not show any measurable adverse reproductive or teratogenic effects in rats fed sodium chlorite in drinking water (Carlton and Smith, 1985). Carlton and Smith (1985) showed that chronic exposure of rats to 100 mg/L sodium chlorite increased the number of abnormal sperms and to some extent, impaired sperm motility. This finding is in contrast to that of Meier and coworkers (1985) who did not detect any sperm-head abnormalities in mice dosed up to 400 mg/L chlorine dioxide, chlorate and chlorite.

The effects of chlorine dioxide on the neurobehavioral development of rats were investigated by Taylor and Pfohl (1985). They observed that administration of chlorine dioxide both postnatal and prenatally resulted in behavioral defects and depressed brain growth. These effects were consistent with depressed thyroid function. The authors also observed

delayed eye opening, decreased body and brain weights and activities, and delayed locomotor activity. Chlorine dioxide, and not chlorite, has been implicated as a hypercholesterolemic agent which increased serum cholesterol levels by interacting with a diet marginal in calcium (Revis et al., 1985). No definitive conclusions have been made on the genotoxicity of chlorine dioxide and its metabolites. Suh et al. (1984) reported that chlorine dioxide depressed DNA synthesis in the testis and intestinal epithelium of rats dosed 100 mg/L chlorine dioxide in their drinking water. Meier et al. (1985) tested the mutagenic activity of chlorine dioxide, chlorate and chlorite in mice using chromosomal aberrations, micronucleus and sperm-head abnormalities assays as endpoints. No mutagenic activity was detected in any of the disinfectants 5 days after exposure. The tumor initiating and promoting activities of chlorate and chlorite have also been investigated. Both chemicals did not demonstrate any significant carcinogenic activity in Sencar mice (Kurokawa et al., 1985; 1984).

With regard to human studies, oral administration of chlorine dioxide, chlorate and chlorite to healthy adult male volunteers did not produce any detrimental clinical effects (Lubbers et al., 1985). The authors employed an exposure time of 12 weeks with the volunteers receiving a total of 5 mg/L chlorine dioxide or its endproducts. Again, no biochemical or physiological effects were observed in the volunteers.

Caution must be taken in interpreting these findings since a lack of effect does not necessarily imply safety. It is not known whether long-term low-dose exposure to any of these chemicals will induce any severe adverse physiological effects.

Mutagenesis and Carcinogenesis

Different classes of compounds have been identified in drinking water, some of which may be produced from chlorination reactions. A 1976 USEPA survey showed that of 289 compounds identified in drinking water, approximately 38% were halogenated. Another survey of five major cities in the United States showed that approximately 50% of the volatile hydrophobic compounds in drinking water were halogenated (USEPA, 1975). The number could be considerably higher if proper analytical methods for detection and quantification were available. Also, the source and distribution of these chemicals differ. This section will briefly review the mutagenic and carcinogenic properties of those compounds that are formed during water chlorination, such as THMs and HANs, and those of precursor substances such as amino acids and humic substances.

Drinking water

The carcinogenic properties of drinking water were first reported by Marx (1974). Since then numerous studies have been conducted on the mutagenic and carcinogenic properties of drinking water in different municipalities in the United

States and elsewhere and, in all cases, researchers have shown that mutagens are produced as a result of water chlorination. Drinking water in the United States and Canada (Basu et al., 1985; Nestmann et al., 1983; Cheh et al., 1980; Loper, 1980), South Africa (Grabow et al., 1980), the Netherlands (Kool et al., 1985), United Kingdom (Horth et al., 1987; Fielding and Horth, 1986), Finland (Kronberg et al., 1988), Israel (Guttman-Bass et al., 1987) and many other countries have been tested and reported to be mutagenic in several short-term assays. In most of the studies, replacement of chlorine with chlorine dioxide leads to a reduction in the observed mutagenic activities (Guttman-Bass et al., 1987). According to the findings of Kool et al. (1985), chlorine treatment of drinking water generally increases the mutagenic activity in S. typhimurium TA98 and TA100. Also chlorine dioxide is capable of increasing the mutagenic activity in certain cases with the overall increase being less than that of chlorine.

The main drawback to these findings is that mutagenicity is usually detected from a mixture of compounds of different classes and origin, and even though compounds such as chloroform, bromoform and tetrachloroethylene are known carcinogens, their overall contribution to the observed genotoxicity is not usually known. Being highly volatile, some of these chemicals may be lost during sample concentration procedures prior to genotoxicity evaluation. The third problem is that some of these compounds are poorly

characterized non-volatiles which cannot be easily isolated and identified, therefore, their overall contribution to the carcinogenic and mutagenic effects remain unknown. None of the studies in the literature has accounted for 100% of the mutagenic activity observed in drinking water extracts. Kronberg et al. (1988) have recently identified a very strong mutagen,3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) and its isomer, (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid (EMX) in acidified chlorinated extracts of humic acid and drinking water. According to the authors, MX accounted for 50-100% of the mutagenic activity of extracts of chlorinated humic water and for 20-50% of the activity in drinking water extracts while EMX accounted for only about 2% of the total mutagenic activities using the Ames Salmonella assay. MX has also been detected in drinking water from three locations in the U.S.A. and in the chlorination of humic acid (Meier et al., 1987). According to the authors, MX appears to account for a significant proportion of the mutagenic activity observed in these samples.

Presented in Table 1 is a of some of the compounds identified in drinking water which includes known or suspected carcinogens. Volatile compounds have been defined as those that are purgeable from drinking water at room temperature with an inert gas such as helium (Tardiff et al., 1978). These compounds represent 5-10% of the total organics in drinking water and the remaining 90-95% have not been fully

Table 1. Some halogenated compounds formed in drinking water as a result of water chlorination[a]

Chloroform[b]	Trichloroacetic acid
Bromodichloromethane[b]	Trichloroacetaldehyde
Chlorodibromomethane[b]	Chlorophenols
Bromoform[b]	Alpha-chloroketones
Dichloromethane	Chlorobenzenes
Bis (2-chloroethyl) ether[b]	Chlorinated acetonitriles[b]
Trichloroethylene[b]	Chlorinated aromatic acids
Tetrachloroethylene[b]	Chlorinated purines
1,1,1-Trichloroethane	Chlorinated pyrimidines

[a]Adapted from Kraybill (1978)

[b]Known or suspected carcinogens

identified, or are unknown (NAS, 1980). Not included are those high molecular condensation and/or polymerization products which are not easily amenable to gas chromatographic analyses even with sample derivatization.

Trihalomethanes and haloacetonitriles

THM were the first class of compounds which aroused interest in chlorination byproduct formation (Rook, 1974; Bellar et al., 1974). Most of the purgeable organics are formed in drinking water at a level of 1 ppb or less. However, chloroform is usually present in both treated and untreated water at much higher concentrations of up to 100 ppb on the average (Tardiff et al., 1978; Bellar et al., 1974). Some of the most frequently encountered chlorinated compounds in groundwater include trichloroethylene, vinyl chloride, tetrachloroethylene, 1,1-dichloroethylene, carbon tetrachloride, 1,1,1-trichloroethane and 1,1-dichlorobenzene (Cotruvo and Vogt, 1985). Chloroform, the major THM, has been shown to be carcinogenic and to increase the incidence of hepatocellular carcinomas and kidney epithelial tumors when administered to rats and mice (NCI, 1976). However, recent studies indicate otherwise (Pereira et al., 1985). In 1983, Pereira showed that THM, including chloroform, possess very little or no tumor initiating activity and that their main action in tumorigenesis is promotion. Pereira et al. showed that chloroform, apart from low level DNA binding, did not possess any tumor initiating property. In a fairly recent

study, Pereira and coworkers (1985) administered 1,800 ppm chloroform to Swiss mice, pretreated with ethylnitrosourea, and reported that chloroform inhibited hepatocarcinomas in the mice. According to the authors, administration of chloroform in the drinking water of Swiss mice inhibited both spontaneous and induced hepatocarcinogenesis caused by ethylnitrosourea. These results are in sharp contrast to the NCI study in which chloroform was shown to be a hepatocarcinogen. In the NCI study, chloroform was administered in corn oil and Pereira and coworkers point out the possibility of a synergistic interaction between the vehicle and chloroform leading to the previously observed hepatocarcinogenicity.

Chloroform has been reported to be non-mutagenic in bacteria assays including Salmonella and E. coli (Simmon et al., 1977; Uehleke et al., 1976). Other THMs such as bromoform, bromodichloromethane and dibromochloromethane were only weakly mutagenic in Salmonella without metabolic activation (Simmon et al., 1977).

The second major class of volatile organics, HANs, have received considerable attention since they were discovered (Oliver, 1983; Bierber and Trehy, 1983). Bierber and Trehy (1983) reported that HANs easily escape detection because they undergo gradual hydrolysis to form nonvolatile products. Dichloroacetonitrile, the major HAN, is usually detected in drinking water at about 10% that of the average THM concentration (Oliver, 1983). Haloacetonitriles (HAN) have

been reported to pose some possible health problems to humans because of their potential mutagenic and carcinogenic properties in test animals (Meier et al., 1983). They have been detected in the stomach contents of rats dosed with aqueous chlorine solutions and the possibility exists for the same effects to be observed in humans (Mink et al., 1983). Bull and Robinson (1985) tested the mutagenic activity of the five common HAN, chloroacetonitrile, dichloroacetonitrile, trichloroacetonitrile, bromochloroacetonitrile, and dibromoacetonitrile in the Ames Salmonella/microsome assay and found that dichloroacetonitrile and bromochloroacetonitrile were direct-acting mutagens which produced base-pair substitution mutations in Salmonella. All five compounds induced significant elevations in sister chromatid exchange (SCE) frequencies in Chinese hamster ovary (CHO) cells. SCE frequencies increased with increasing chlorine substitution and was further enhanced when bromine was substituted for chlorine. Three of the compounds were also capable of producing tumors in Sencar mice. HAN have been shown to bind covalently to liver and kidney DNA and cause DNA strand breaks, which indicates potential carcinogenic ability (Pereira et al., 1985). Trichloroacetonitrile was found to be the most potent DNA strand breaker while the bromo-derivatives were intermediate. Pereira and coworkers have proposed a scheme for HAN metabolism which involves formation of phosgene, a highly reactive electrophile, and cyanide.

The above findings were further confirmed by Daniel et al. (1986) who reported that HANs were cytotoxic in human cultured lymphoblast (CCRF-CEM) cells. They produced 13-60% cell killing at a dose of 50 μmol and caused major DNA strand breaks. It should be noted that in all of these studies, chlorine dioxide has not been mentioned due to the fact that its use in water treatment does not lead to THM formation. Further studies are needed to determine the stability of HANs in aqueous media and the possible health hazards associated with their long-term low-dose exposure to humans.

Precursor substances

The final part of this review will focus on those precursor substances which react with chlorine or chlorine dioxide to form genotoxicants. Proteinaceous material and humic and fulvic substances are known precursors of haloacetonitrile and trihalomethane formation in natural waters upon reaction with chlorine. Unfortunately, these compounds are not the only ones of concern in drinking water since other chlorinated and non-chlorinated byproducts are formed as well. Even though chlorine dioxide produces very little or no THM in water, it does not remove them from water. Also, some of the oxidation byproducts of chlorine dioxide reactions in water may also be genotoxic.

Amino acids, peptides and proteins. The genetic effects of amino acids after chlorination were first reported by Sussmuth (1982) who demonstrated that harmless nutrients such as amino

acids and yeast and meat extracts could be converted into carcinogens and mutagens by chlorination. In this experiment, 80 mL of chlorine gas were bubbled through 250 mg of yeast extract, meat extract or amino acids for 4 minutes and the reaction products evaluated for genotoxic activity. Due to the high chlorine dose, Sussmuth did not find the need to concentrate the reaction products. Methionine was found to be the most potent precursor of genotoxic activity both in the Ames Salmonella assay and the rec assay in Bacillus subtilis. Tyrosine, phenylalanine, cysteine and glycine were also genotoxic. In the mutagenicity assay, all the above compounds were direct-acting mutagens which did not require metabolic activation. None of the reaction products were identified in this study.

In another study, Horth et al. (1987) evaluated the mutagenic activity of several amino acids in Salmonella after reaction with sodium hypochlorite. With the exception of glycine, mutagenic activity was detected in all the amino acids treated with chlorine. Methionine, phenylalanine, tyrosine and a group of heterocyclics were found to be the most potent precursors of mutagenic activity. Of the heterocyclics, tryptophan and proline were the most active. The authors identified several compounds in their mutagenic extracts. Except for dichloroacetonitrile which is a known carcinogen, none of the other compounds identified possessed mutagenic activity when tested separately. It was concluded

that the level of dichloroacetonitrile was too low and unlikely to account for any significant portion of the mutagenicity. In follow-up studies Horth and coworkers (1989) tentatively identified several other compounds from chlorination of tyrosine and phenylalanine. Using authentic standards, the authors showed that dichloroacetonitrile, produced from both amino acids, and benzylchloride and 2-phenyl-2,2-dichloroethenal, produced from phenylalanine, were all mutagenic to S. typhimurium strain TA100. Dichloroacetonitrile has also been shown to be produced from the chlorination of aspartic acid, tyrosine and tryptophan (Trehy et al., 1986). The overall contribution of the unidentified compounds and those for which no authentic standards were available still remain unknown.

In similar studies Rapson and coworkers (1985) tested the mutagenicity of compounds produced by reaction of aqueous chlorine and chlorine dioxide with tyrosine, at increasing chlorine to tyrosine ratios. Tyrosine produced mutagenic reaction products with the mutagenicity increasing with chlorine dose. Mutagenicity peaked at 4 equivalents of chlorine per mole of tyrosine. Substitution of chlorine with chlorine dioxide led to a linear decline in mutagenic activity from 2,100 revertants over background to 0 with 100% chlorine dioxide. Similar results were obtained by Tan et al. (1987b) who showed that replacement of chlorine with chlorine dioxide on a molar basis decreased the mutagenic activity in

chlorinated tryptophan concentrates. However, in the case of two dipeptides, L-tryptophylglycine and L-glycyl-L-tryptophan, reaction with chlorine dioxide led to the formation of mutagens while aqueous chlorine did not (Tan, 1986). In contrast to the findings of Sussmuth (1982) and Horth et al. (1987), Tan et al. (1987b) did not detect any mutagenic activity after reaction of aqueous chlorine and methionine.

Chlorination of purines and pyrimidines as well as nucleosides and nucleotides did not result in any mutagen formation (Horth et al., 1987). It is evident that many difficulties exist in the concentration, fractionation and evaluation of individual components in drinking water or model systems. In a majority of tests, a mixture of compounds and not individual components are used due to analytical difficulties encountered sample separation. Unless proper separation and analytical methods are developed, it will be difficult to pinpoint the individual chemical or group of chemicals responsible for mutagenic activity in chlorination reaction mixtures.

Fulvic and humic substances. Fulvic and humic acids are naturally occurring metabolic endproducts of decaying vegetation which account for the bulk of organic matter present in surface waters. Chlorination of water containing these substances has been reported to result in the formation of THMs and other non-chlorinated, high molecular weight, poorly characterized organics (Coleman et al., 1983; Rook,

1977), some of which are mutagens and carcinogens (Kronberg et al., 1988; Meier et al., 1987, 1986 and 1983).

Chlorination of kraft wood pulp, catechol, lignin and humic and fulvic acids have all been shown to give rise to mutagen formation in the Ames Salmonella/microsome assay (Kronberg et al., 1988; Meier et al., 1987; Holmbom et al., 1984; Kringstad et al., 1983; Meier et al., 1983; Nazar and Rapson, 1982; Nazar et al., 1981). The pH of the reaction is important in mutagen formation especially when Amberlite XAD resins are used to adsorb or concentrate the reaction mixtures. According to Nazar and Rapson (1982), at elevated pH of 7 or higher, mutagenic activity decays due to the cleavage of organically bound chlorine by hydroxide ions. These findings have been confirmed by Meier et al. (1983) who observed a decrease in mutagenic activity with increasing pH due in part to the alkali lability of the compounds. Mutagenic activity of chlorinated model humic acids is similar to that of drinking water which implies that these substances account for a substantial portion of the mutagenic activity of drinking water (Kronberg et al., 1988; Meier et al., 1987; Coleman et al., 1984). Also most of the mutagenic activity is due to non-volatile compounds since most of the volatile organics are lost during sample concentration (Meier et al., 1983). Coleman et al. (1984) identified more than 25 compounds from ether extracts of chlorinated humic acids and confirmed their individual mutagenic activities using

authentic standard compounds. Kronberg and colleagues have also identified and characterized a strong mutagen, MX, from acidified extracts of chlorinated humic acid using HPLC fractionation techniques. MX accounted for 50-100% of the observed mutagenic activity while its isomer, EMX, accounted for very little of the observed activity. In a separate study, Meier et al. (1986) identified several compounds from ether extracts of chlorinated humic acids, some of which are known mutagens. Analysis of these extracts indicated that they accounted for only 7% of the total activity in the original solutions. They also identified MX and EMX which had previously been identified (Holmbom et al., 1984). Further studies by Meier et al. (1987) and Hemming et al. (1986) showed that MX accounted for between 15-34% and 3-19%, respectively, of the mutagenic activity in XAD concentrates of drinking water. Both research groups observed that sample acidification prior to XAD adsorption was essential for the effective recovery of MX from reaction drinking water or chlorination reaction mixtures.

In summary, evidence has been provided to show that genotoxicants are produced during chlorination of water which contains proteinaceous material and fulvic and humic substances. A comparison of the mutagenicities of chlorinated and non-chlorinated surface waters has always shown that mutagens are present in the chlorinated samples. In any environmental impact assessment study, those compounds that

are not easily identifiable should be taken into account in order to ensure that they are not being ignored.

CHAPTER III
MATERIALS AND METHODS

Chlorine Demand-free Water

Chlorine demand-free water was prepared following a modification of the procedure of Ghanbari et al. (1982a). Distilled water was passed through two successive Barnstead deionizing units (Barnstead, Division of Sybron Corporation, Boston, MA) and then through a glass column containing Porapak Q (Supelco, Inc., Bellefonte, PA). The water was analyzed by iodometric titration (APHA, 1985) to determine the presence of any available chlorine. Water used for the preparation of all the reagents and substrates was prepared this way.

Generation of Aqueous Chlorine

Commercial chlorine is contaminated with a variety of organochlorine compounds including chloroform, phosgene, carbon tetrachloride, methylene chloride, etc. (Christman, 1983). To ensure that no such contaminants were present in the chlorine sample used in this study, a modification of the methods of Ghanbari et al. (1983) was used for the generation of aqueous chlorine. Chlorine gas was prepared by dropwise addition of 3 N HCl to $KMnO_4$ (Fisher Scientific, Fair Lawn, NJ) in a closed system under vacuum. The liberated gas was

trapped in ice-cold chlorine demand-free water or 0.1 M sodium phosphate buffer, pH 7 as desired. Under these generation conditions, the gas was efficiently trapped mainly as hypochlorous acid. This was detected by a drop in the pH of the solution from 7.0 to 2.5-3 due to the liberation of hydrogen ions. The purity and available chlorine concentrations of the stock solutions were determined by iodometric titration (APHA, 1985). Aqueous chlorine solutions were diluted when necessary and used immediately after preparation.

Generation of Aqueous Chlorine Dioxide

Stock solutions of aqueous chlorine dioxide were prepared by slow addition of 2 N H_2SO_4 to 6 g sodium chlorite (Eastman Kodak Co., Rochester, NY) in 6 mL water in a closed system (APHA, 1985). The chlorine dioxide gas generated from the reaction was passed through a sodium chlorite clean-up column to remove any contaminants such as chlorine. The gas was then collected as a greenish-yellow solution in ice-cold chlorine demand-free water or 0.1 M sodium phosphate buffer as desired. As before, the concentrations of chlorine dioxide solutions were determined iodometrically. To prevent losses due to volatilization and/or breakdown, chlorine dioxide solutions were always prepared fresh, diluted when necessary, and used immediately after preparation. All chlorine and chlorine

dioxide stock solutions were prepared under yellow lights to prevent photodecomposition.

Iodometric Titration

Iodometric titration was used to determine available chlorine and chlorine dioxide concentrations in all the stock solutions as well as chlorine residuals remaining after the reactions. The method involves liberation of iodine from potassium iodide (KI) by chlorine. The liberated iodine is then titrated with sodium thiosulfate ($Na_2S_2O_3$) to a starch end point (APHA 1985). Excess KI acidified with acetic acid to pH 3-4 was mixed with aliquots of the stock chlorine or chlorine dioxide solutions in glass-stoppered flasks at room temperature. In the case of chlorine dioxide solutions, the chlorine dioxide-KI solutions were kept in the dark for about 5 minutes prior to titration. The mixtures were then titrated under yellow lights with 0.01 N $Na_2S_2O_3$ solution prepared by diluting from standardized stock solutions, until the yellow color of the liberated iodine was almost clear. A few drops of a 0.5% starch indicator solution were added and titration was continued to a colorless end point. A blank titration using only chlorine demand-free water was also performed to correct for interferences from oxidizing and reducing substances and to correct for the concentration of iodine bound starch at the end point (APHA, 1985).

The total available chlorine or chlorine dioxide was calculated as follows:

$$\text{mg Cl or } ClO_2 \text{ as } Cl_2/L \text{ (ppm)} = \frac{(A \pm B) \times N \times 35450}{\text{mL sample}}$$

where: A = Volume of $Na_2S_2O_3$ used for the sample,

B = Volume of $Na_2S_2O_3$ used for the blank,

N = Normality of the $Na_2S_2O_3$ used.

Reactions of Aqueous Chlorine or Chlorine Dioxide with L-Tryptophan

Preliminary reactions of aqueous chlorine

In preliminary experiments, 0.1 M L-tryptophan (Sigma Chemical Co., St. Louis, MO) solutions were prepared in 0.1 M sodium phosphate buffer at pH 7.0 with slight warming to ensure that all the amino acid was dissolved. Two molar ratios of aqueous chlorine to amino acid, 1:1 and 3:1, and a reaction time of 24 hours was used to ensure that the reaction was complete. This study used aqueous chlorine alone with pH adjustment of the reaction mixture after 24 h to obtain acidic, neutral and basic fractions. Each fraction was extracted separately first with hexane, then with methylene chloride and lastly with ethyl ether.

Reactions of aqueous chlorine and chlorine dioxide

In the next series of experiments, three molar ratios of L-tryptophan to aqueous chlorine or chlorine dioxide, 1:1, 1:3 and 1:7 (M:M), were chosen. Tryptophan solutions were

prepared in 0.1 M sodium phosphate buffer, pH 7.0, as before and aqueous chlorine and chlorine dioxide solutions were prepared in chlorine demand-free water. For the 1:1 and 1:3 reactions, 14 mM tryptophan solutions (approximately 2.86 g tryptophan in 1 L sodium phosphate buffer) were reacted with 14 and 42 mM chlorinating agents (total volume 1 L), respectively. The 1:7 reactions were carried out using a 10 mM amino acid solution (2.04 g tryptophan in 1 L sodium phosphate buffer) and a 70 mM chlorine or chlorine dioxide (total volume 1 L). The amino acid concentration used for the latter reactions were limited by the amount of chlorinating agent generated by the above procedures. All the reactions were carried out using equal volumes of reagent and substrate (2 L final volume), therefore; the final concentrations of chlorinating agents and amino acid were halved. Reactions were carried out in the dark in glass jars at ambient temperature for 24 hours with continuous mixing on a Corning stirrer. Procedural blanks containing only amino acid, aqueous chlorine or chlorine dioxide in sodium phosphate buffer were used as controls.

After reaction, the samples were filtered using a 0.45 μm membrane filter (Gelman Sciences, Inc., Ann Arbor, MI) to remove any precipitate present. Aliquots of the reaction mixture were titrated iodometrically to determine if any available chlorine remained. The samples were then split in half. The pH of one-half was readjusted to 2.5 with 85%

phosphoric acid and saved for resin adsorption, while the other fraction was kept at neutral pH for liquid-liquid extraction using ethyl ether.

Chlorine Consumption

Chlorine concentrations before and after chlorination were measured iodometrically (APHA, 1985). Any excess chlorine or chlorine dioxide remaining after the 24-hour reaction was quenched using sodium sulfite (Fisher Scientific, Fair Lawn, NJ) in order to avoid contamination with elemental sulfur produced from dechlorination with sodium thiosulfate (Trehy et al., 1986).

Concentration Techniques

Different techniques, mainly liquid-liquid extraction, Amberlite XAD adsorption and column chromatography were used to concentrate the reaction mixtures for mutagenicity and other assays.

Liquid-liquid extraction

Preliminary studies. In preliminary liquid-liquid extraction studies, the active ingredients in the aqueous chlorination reaction mixtures were extracted using organic solvents of different polarity. Ethyl ether and hexane (Fisher Scientific) were reagent A.C.S. and methylene chloride (Fisher Scientific) was HPLC grade. The reaction mixtures containing aqueous chlorine and tryptophan at neutral pH were extracted

three times by mixing vigorously on a stirrer with equal volumes of hexane, followed by methylene chloride and then ethyl ether. Each extraction took approximately 2 hours and represented the neutral fraction. After these extractions, the aqueous mixture was divided into two portions. The pH of one fraction was adjusted to 1.2 using concentrated HCl and that of the other half was changed to pH 11.7 using 10 N sodium hydroxide. These fractions were each extracted with the three organic solvents as before to obtain acidic and alkaline fractions, respectively, as shown in Fig. 5.

In the preliminary studies, high mutagenic activity was detected in the ethyl ether extracts of the reaction products. Therefore, in the latter part of the study when three ratios of aqueous chlorine and chlorine dioxide were compared, only ethyl ether was used for the liquid-liquid extractions. Mixtures containing different molar ratios of aqueous chlorine or chlorine dioxide with tryptophan were extracted vigorously with mixing on a stirrer with the aid of a magnetic stirrer for approximately 4 hours, and then separated with a separatory funnel. The aqueous phase was discarded and the ethyl ether extract was dried by rotary evaporation and saved for further analysis.

Evaluation of product distribution in the ethyl ether extracts. A solution containing radiolabeled ^{14}C-tryptophan of known activity was prepared as described below. The reaction products were extracted with ethyl ether for 2 hours.

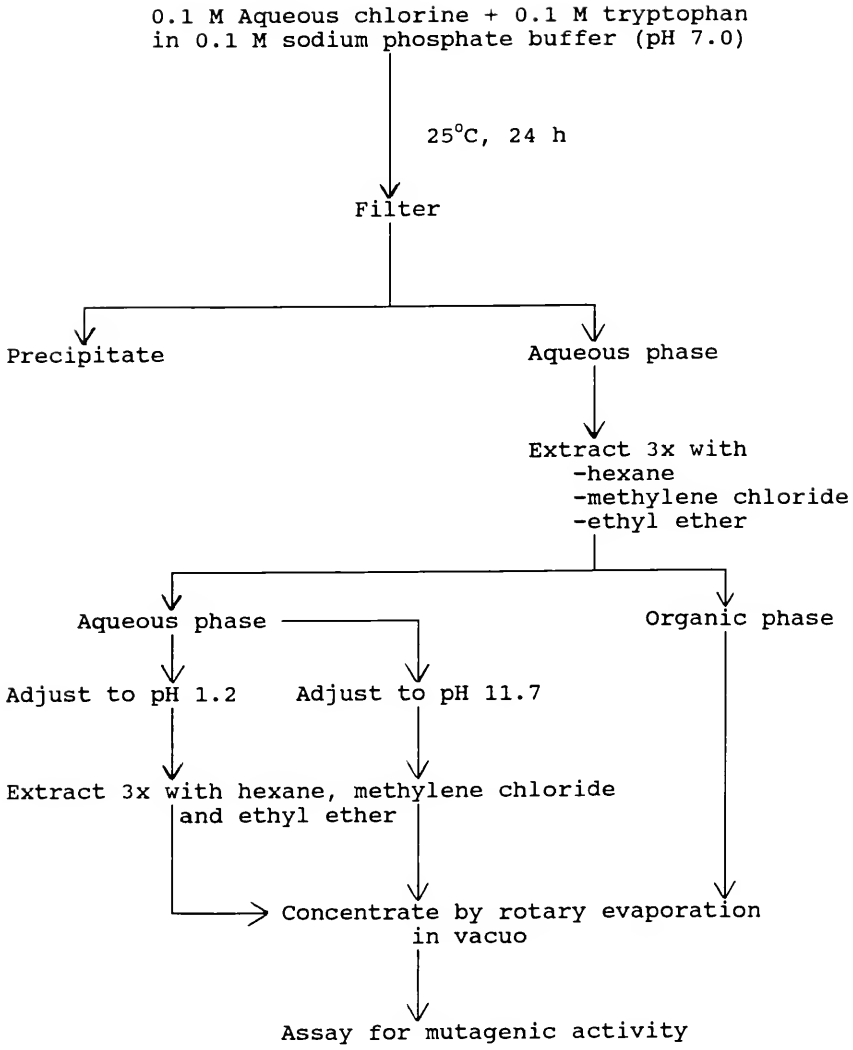

Figure 5. Schematic presentation of sample preparation for mutagenicity assessment in the preliminary studies

The ethyl ether layer was separated from the aqueous layer and partially dried to a known volume. The percentage distribution of radioactivity in the aqueous solution and ether extracts was measured by dissolving aliquots in 15 mL Scintiverse II cocktail and counted on a Beckman Instruments liquid scintillation spectrometer as described below.

Amberlite XAD polymeric resin adsorption

Evaluation of Amberlite XAD procedure for concentrating the reaction products. Amberlite XAD-2, a nonpolar polystyrene adsorbent and XAD-8, an acrylic ester of intermediate polarity, were obtained from Rohm and Haas (Philadelphia, PA). The resins were prepared prior to use by continuous soxhlet extraction with acetone for 5 hours followed by further washing with methanol for an additional 5 hours. The clean resins were stored separately in methanol until needed. Prior to use, the resins were slurry packed in methanol in a glass column, 52 cm by 1.9 cm i.d. to form a bed height of 30 cm. The resins were then rinsed with 3-5 bed volumes of distilled water to ensure that all the methanol was removed.

A 7 mM tryptophan solution in 0.1 M sodium phosphate buffer (pH 7.0) was prepared by dissolving 0.5 g of the amino acid in 350 mL buffer. Radiolabeled L-tryptophan (side chain-3-^{14}C) with a specific activity of 53.76 mCi/mmole was obtained from New England Nuclear, Inc. (Boston, MA). Radiolabeled tryptophan (0.1 mCi in 0.1 mL phosphate buffer) was mixed with

the tryptophan solution and the total radioactivity of the solution was measured. Equimolar concentrations of either aqueous chlorine or chlorine dioxide were added to separate reaction flasks and allowed to react for 4 hours in the dark at room temperature. The reaction mixtures were filtered separately using a 0.45 μm membrane filter and the pH and radioactivity of the solutions were measured. The solutions were then passed through a glass column (52 cm x 1.9 cm i.d.) containing either XAD-2 alone or in combination with XAD-8 (1:1 mixture) at a bed height of 30 cm.

Trial experiments were conducted to determine the best Amberlite XAD concentration technique for the reaction products. In one experiment, XAD-8 was placed on top of XAD-2 while in another the order was reversed. In another trial, only XAD-2 was used. Also, the importance of sample pH on the recovery of the reaction products was evaluated by adjusting sample pH. The pH of the reaction products in one experiment was adjusted to 2.5 with 85% phosphoric acid while in another, it was maintained at 7.0. All the reaction products were passed through the various resin columns at a flow rate of approximately 40 mL/minute. The first pass effluent was poured on the column. The adsorbed substances were eluted successively with 200 mL each of methanol, ethyl ether and acetone. The combined organic solvents were removed by rotary evaporation under vacuum and the residues were diluted to 10 mL with chlorine demand-free water. Radioactivity of the

aqueous filtrates and concentrates were measured by counting 0.1 mL aliquots in 15 mL Scintiverse II cocktail (Fisher Scientific, Fair Lawn, NJ) using a Beckman Instruments spectrometer Model LS 2800 (Beckman Instruments, Inc., Fullerton, CA) with automatic external standardization. Distribution of radioactivity in the filtrates and eluates was used to measure the recovery of the reaction products. Similar sample preparation was used to determine product distribution in the liquid-liquid extractions.

Due to the high mutagenic activity in the Amberlite XAD-2/8 acetone eluates of the 7:1 (aqueous chlorine or chlorine dioxide:tryptophan) ratio, radioactivity distribution in these eluates were also determined.

Sample concentration for mutagenicity assessment using Amberlite XAD resins. Based on the results from the preliminary studies, a resin column with Amberlite XAD-8 placed on top of XAD-2 (1:1 mixture) was used for concentrating the reaction products. The pH of the aqueous mixtures containing the reaction products of either chlorine or chlorine dioxide with tryptophan was adjusted to 2.5 with 85% phosphoric acid and passed through the resins at a flow rate of approximately 40 mL/minute. This procedure was repeated using the first-pass effluent. Control samples containing aqueous chlorine, chlorine dioxide or tryptophan alone in sodium phosphate buffer were dechlorinated with sodium sulfite and also concentrated by resin adsorption.

After most of the aqueous fraction had passed through the column and the water level was barely on top of the resin, acetone was added and allowed to flow freely through the column until it reached the bottom. Flow was interrupted and the acetone allowed to equilibrate with the resins for 10-15 minutes. A total of 300 mL of acetone was used to elute most of the adsorbed substances from the column. Any color or substances remaining on the column was further eluted successively with 300 mL methanol followed by 300 mL ethyl ether. All the eluates were concentrated separately by rotary evaporation (Fig. 6). The dry samples were weighed, dissolved in and diluted with spectrophotometric grade dimethyl sulfoxide (DMSO) and aliquots used for the mutagenicity studies.

Rotary evaporation

The rotary evaporator used was a Buchi Rotavapor R 110 (Brinkmann Instruments, Inc., Westbury, NY). The aqueous reaction mixtures containing tryptophan and chlorine as well as all the organic solvents obtained from the liquid-liquid extractions and Amberlite XAD adsorptions were concentrated by rotary evaporation *in vacuo* at 45°C.

Concentrated samples were quantitatively transferred into preweighed teflon-capped vials and the remaining traces of organic solvents were removed under a gentle stream of nitrogen gas. Samples were then weighed, dissolved in and diluted with spectrophotometric grade DMSO and used for the

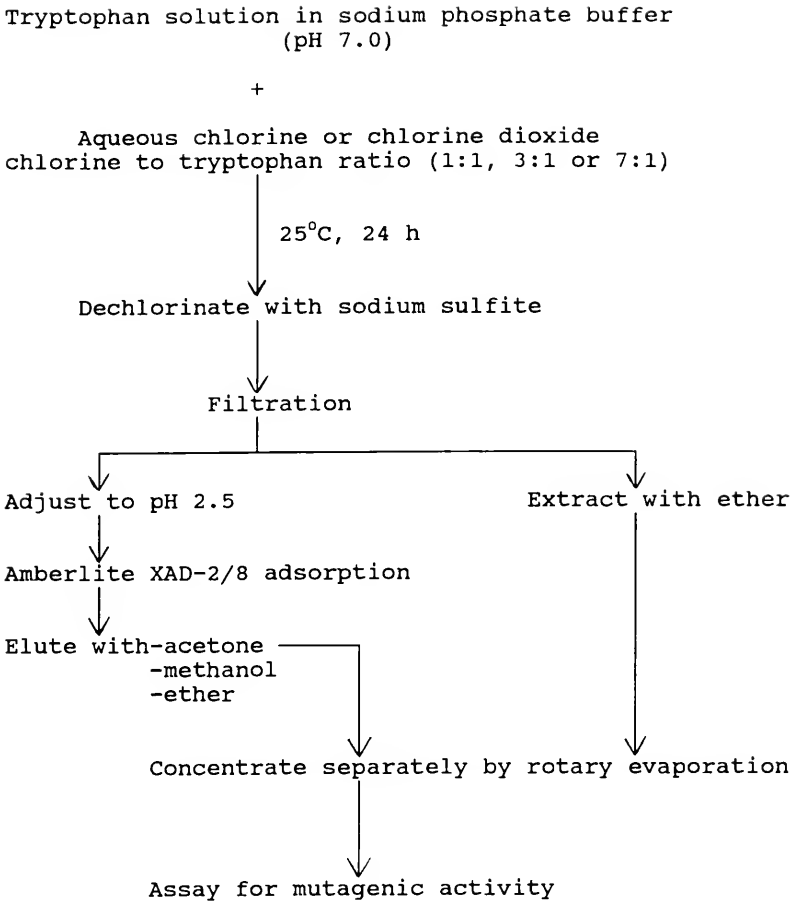

Figure 6. Schematic presentation of sample preparation for mutagenicity assessment in the reactions of aqueous chlorine or chlorine dioxide with tryptophan

Ames mutagenicity assay. Those XAD samples containing water and the aqueous concentrates were diluted in chlorine demand-free water, aseptically filtered and aliquots used for the mutagenicity assay.

Fractionation of Reaction Product Extracts

Highly mutagenic liquid-liquid extracts as well as Amberlite XAD resin concentrates were further fractionated using thin layer chromatography (TLC).

Thin layer chromatography

Uniplate silica gel G (Analtech, Inc., Newark, DE) and Fisher brand Redi/Pit Sil gel G (Fisher Scientific), both 20 x 20 cm, were used for TLC separation. Prior to use, the TLC plates were heat-activated for 1 hour at 150°C and allowed to cool in a Brinkman Instruments (Westbury, NY) relative humidity holding chamber before use. The thin Fisher brand qualitative plates were used for preparative sample fractionation and solvent evaluation to determine the best solvent combinations and ratios for resolving the extracts into single bands. A solvent system containing hexane, ethyl ether and/or methanol acidified with acetic acid was found to be suitable for developing the plates.

The thick Analtech silica gel plates were used for sample preparation prior to genotoxicity evaluation. Concentrated samples, approximately 1 mL, were applied as a thin band across the length of the plate. The aqueous chlorine-treated

extracts were developed using hexane:ethyl ether:acetic acid (50:50:2, v/v/v) while chlorine dioxide-treated samples were developed with hexane:ethyl ether: methanol:acetic acid (63:30:7:2, v/v/v/v). In all experiments, a total of about 3 mL of sample was fractionated this way for the mutagenicity assessment. The solvent systems were freshly prepared for each run and the TLC plates were allowed to develop in the dark for about 0.75-1 hour, usually resulting in 13-14 cm movement of the solvent front. The plates were then air-dried and examined for fluorescence under a Chromato-Vue cabinet model CC-20G (Ultra-Violet Products, Inc., San Gabriel, CA) using long wavelength UV detection. A band from the aqueous chlorine and tryptophan reaction products was re-developed on another TLC plate by using a mobile phase containing hexane: methylene chloride:acetic acid (50:50:2, v/v/v).

Any bands obtained were scraped off with a spatula, extracted with acetone and filtered through a Whatman No. 4 qualitative filter paper. As before, all extractions were performed under yellow lights to ensure that any photosensitive materials were not destroyed. The filtrate was finally concentrated by rotary evaporation, weighed and dissolved in DMSO and used for genotoxicity evaluation. The stability of the TLC extracts was also checked by leaving some plates at room temperature for up to 48 hours and monitoring their long wavelength UV absorbance.

Product distribution in the TLC fractions. Aliquots from the radioactive reaction products (7:1 ratio) were spotted on TLC plates and developed with the solvents for aqueous chlorine and chlorine dioxide as described above. The fluorescent bands were scraped off and dissolved in Scintiverse II cocktail solution. To ensure that all the radioactivity was accountered for, the gaps between the fluorescent samples all the way to the solvent front were also scraped off individually and dissolved in Scintiverse II cocktail. Radioactivity in all the samples including silica gel blanks was counted as previously described.

Genotoxicity Assays

The mutagenic potentials of all the extracts and subfractions were evaluated using the Ames Salmonella/ mammalian microsome assay (Ames et al., 1975). The highly mutagenic TLC subfractions were further evaluated for genotoxic effects using the sister chromatid exchange (SCE) assay in vitro in Chinese hamster ovary (CHO) cells.

Ames Salmonella/mammalian microsome assay

The standard methods developed by Ames et al. (1975) and later modified by Maron and Ames (1983) were used to determine the mutagenic activity of the reaction products. Salmonella typhimurium strains TA98 and TA100 were generously provided by Dr. Bruce N. Ames (University of California, Berkeley, CA).

The bacteria were grown in nutrient broth (Nutrient Broth No. 2, Oxoid Ltd., Hants, England) and stored as described by Maron and Ames (1983). The tests were performed with and without the rat hepatic S-9 mix. The liver S-9 fraction was prepared from the livers of male Sprague-Dawley rats induced with Aroclor 1254 according to the recommendations of Maron and Ames (1983). The rat liver S-9 preparation was thawed immediately before use and mixed with an NADPH-generating system comprising 4 mM NADP, 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl and 100 mM sodium phosphate (pH 7.4). This system provided a broad spectrum of xenobiotic metabolizing enzymes capable of activating or inactivating the test compounds.

For the regular plate incorporation assay, 0.1 mL of a fresh overnight bacterial culture (approximately 1 x 10^8 cells) was added together with predetermined doses of the test compounds to 2.5 mL of soft top agar containing 10% of 0.5 mM L-histidine.HCl and 0.5 mM biotin (Sigma Chemical Co., St. Louis, MO). In tests requiring metabolic activation, 0.5 mL of the S-9 mix was also added, while in tests without metabolic activation it was replaced with 0.5 mL of 0.25 M sodium phosphate buffer (pH 7.4). The resulting liquid suspension was thoroughly mixed and poured onto petri dishes with Vogel-Bonner agar medium containing sources of carbon and nitrogen for the growth and expression of revertant colonies.

Concurrent positive and solvent controls were included in all experiments to confirm desired reversion properties of each strain of bacteria and also S-9 activity. In the presence of the S-9 mix, 2-aminofluorene (2-AF, Aldrich Chemical Co., Inc., Milwaukee, WI) was used as the positive control for both strains of bacteria while 2-nitrofluorene (2-NF, Aldrich Chemical Co.) and methyl methanesulfonate (MMS, Aldrich Chemical Co.) were used for TA98 and TA100, respectively, when the S-9 mix was absent. Solvent controls, mainly DMSO, S-9 mix and sodium phosphate buffer (pH 7.4), were tested without the addition of test compounds in order to determine the number of spontaneous colonies for both tester strains. Four plates were used for each strain and dose of test chemical or controls and each test sample was assayed at least twice. The plates were incubated in the inverted position in a Gravity convection incubator (Precision Scientific, Chicago, IL) at 37°C for 48 hours. The number of revertant colonies was counted and recorded as a function of volume or weight of test sample assayed. The general protocol complied with the workshop recommendations on the Ames Salmonella/microsome assay regarding basic techniques such as strain maintenance, genotypic characteristics checks, S-9 preparation and the examination of the background lawn for toxicity (de Serres and Shelby, 1979). The mutagenicity ratio, the ratio of the number of revertants per test sample divided by that of the controls, was used for statistical

analysis. A ratio of 2 or more is generally considered positive in this assay (Ames et al., 1975). However, a modification of the Student's t-test was used to determine statistical significance (Steel and Torrie, 1980).

Sister chromatid exchange (SCE) assay

Chinese hamster ovary ($CHO-K_1-BH_4$) cells were kindly provided by Dr. Abraham W. Hsie, Oak Ridge National Laboratory, Oak Ridge, TN. The cells were grown as monolayers in Corning T75-cm^2 flasks in a Narco incubator (Portland, OR) with humidified atmosphere of 5% CO_2 in air at 37°C, in Eagles medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin-streptomycin (Gibco Laboratories). To maintain karyotypic stability, the cells were used no more than 15 passages after they were received. Cells were usually retrieved from frozen samples stored in liquid nitrogen and maintained as before.

The assays were performed without the metabolic activation enzyme system from rats pretreated with Aroclor 1254. The metabolic activation system was omitted because results from the previously mentioned Ames mutagenicity assay indicated that it was not required for mutagenic activity. For the SCE assays cells were grown in Corning T25-cm^2 flasks. One day after culture inoculation, medium was replaced and the exponentially-growing cells (about 4×10^5 cells/T25-cm^2 flask) obtained by counting aliquots of the cell suspension on a hemacytometer, were treated with different concentrations of

the test chemicals and controls for 2 hours to ensure chemical interaction with the cells. Eagles basal medium and MMS were used as negative and positive controls, respectively. After the exposure period, the media were removed and the cells were washed twice with phosphate buffered saline (PBS) to remove any traces of test chemical present. Fresh medium containing 5-bromodeoxyuridine (BrdU, 3 μg/mL, Sigma Chemical Co.) was added and incubation was continued for an additional 22 hours. Cultures were wrapped in aluminum foil and maintained in the dark with colchicine (0.1 μg/mL, Sigma Chemical Co.) being present for the final 2-3 hours of culture.

Chromosome preparations

Three hours after addition of colchicine, cells were harvested by mitotic shake-off (Terasima and Tolmach, 1961) and swollen for 20 minutes with a hypotonic solution (75 mM KCl). Cells were then fixed twice by washing for 20 minutes each with a non-aqueous fixative (3:1 methanol:glacial acetic acid, v/v), then dropped from a distance of 10-15 cm onto clean cold slides to rupture the cells, and then air-dried.

A modified fluorescence plus Giemsa technique (Goto et al., 1978; Perry and Wolff, 1974) was used for differential staining of sister chromatids for SCE analyses. Slides were stained for 10 minutes in fluorochrome Hoechst 33258 (5 μg/mL, Calbiochem-Behring, La Jolla, CA) in Gurr buffer (pH 6.8), rinsed and mounted in the same buffer, and exposed at 60°C for 1 hour to a UV light source. Slides were rinsed in distilled

water, stained in 4% Wright-Giemsa (Diagnostic Systems, Inc., Gibbstown, NJ) in Gurr buffer (pH 6.8) and permanently mounted in Pro-Texx (Lerner Laboratories, New Haven, CT) mounting medium. Selection of cells for scoring was based on proper morphology, good spread and a chromosome number of 21 ± 2 (Galloway et al., 1985). SCE frequencies were subjected to square root transformation to equalize the variances (Latt et al., 1981) and analyzed statistically using the Dunnett's test (Steel and Torrie, 1980). A total of 50 metaphases was scored (25/replicate dose) for each dose tested.

Gas Chromatograph/Mass Spectrometer/Data System

A Finnigan Instruments (Sunnyvale, CA) automated combination quadrupole mass spectrometer (Model 4500)/gas chromatograph/data system (Incos Rev. 5.4) was used to identify some of the mutagens present in the chlorinated extracts. Mass spectra of solid samples were obtained by use of the solid probe direct inlet source. Mass spectra of compounds in the various extracts fractionated by TLC and some liquid-liquid extracts were obtained and compared to the spectra of known compounds in the spectral library. A DB-5 capillary column (30 m x 0.32 mm i.d., J. and W. Scientific, Rancho, Cordova, CA) was programmed linearly from 50-300°C at a rate of 12°C/minute with helium as the carrier gas. Helium carrier gas flow rate was maintained at 25 mL/minute. Chromatograms were run in both positive chemical ionization

(CI) and electron impact (EI) modes with methane as the reagent gas. Electron energies of 70 eV were used to generate total ion current chromatograms and recorded mass spectra.

CHAPTER IV
RESULTS

Mutagenicity of the Reaction Products of Aqueous Chlorination of L-Tryptophan

Aqueous chlorine reacted readily with different molar concentrations of tryptophan at ambient temperature to produce a dark-colored precipitate. The amount of precipitate formed increased linearly with time and levelled off after about 5 days. When the molar concentration of aqueous chlorine was increased more than 15-fold over that of the amino acid, color formation was not observed.

Rotary evaporation

Preliminary studies were conducted to confirm the mutagenic activity of chlorination reaction mixtures of aqueous chlorine and tryptophan and to find the best method to concentrate and fractionate the reaction products for genotoxicity assessment. In one study, a reaction mixture containing equimolar concentrations of aqueous chlorine and amino acid (0.05 M final concentration) was concentrated about 10-fold by rotary evaporation under vacuum at 55°C. The concentrate was filtered to obtain a precipitate and a brown to light-brown filtrate. The filtrate was filter-sterilized using a 0.45 μm membrane filter and the precipitate was

dissolved in DMSO and assayed for mutagenic activity. Procedural blanks containing only tryptophan were also concentrated to serve as controls. The results of this study are presented in Tables 2 and 3. For statistical analyses of all the mutagenicity data, the mutagenicity ratio, the ratio of the number of revertants per test dose (spontaneous and induced) divided by that of the respective negative controls (spontaneous revertants), was used. A mutagenicity ratio value of 2 or more is generally considered as a statistically significant indication of mutagenic activity (Ames et al., 1975). Also, a modification of the Student's t-test was used to determine statistical significance (Steel and Torrie, 1980).

The aqueous concentrate exhibited mutagenic activity towards both strains of S. typhimurium TA98 and TA100 at all the doses tested (Tables 2 and 3). There was a decrease in the number of revertants at the highest doses tested. This is due to the fact that at these high levels, the compounds were beginning to show some toxicity to the bacteria. Addition of the rat hepatic S-9 mix to the test system led to a decline in mutagenic activity in strain TA100 at all the doses tested (Table 3). In bacterial strain TA98, there was a reduction in the mutagenic activity at the lower doses and an increase in activity at the highest dose tested when the S-9 mix was added (Table 2). In the latter, addition of the

Table 2. Mutagenicity of the aqueous concentrate arising from the reaction of equimolar concentrations of aqueous chlorine and tryptophan using Salmonella typhimurium strain TA98[a]

		Number of revertants/plate[b]			
		TA98			
Sample	Dose/plate[c]	-S9	MR[d]	+S9	MR
Bacteria		26± 3		28± 7	
Bacteria + DMSO	25 μL	24± 2		30± 7	
Aqueous fraction	100 μL	225±44	8.7	33± 6	1.2*
	200 μL	338±30	13.0	62± 30	2.2*
	300 μL	256±27	9.8	381±115	13.6
Precipitate	1 mg	41± 5	1.7	36± 6	1.2*
	5 mg	45± 8	1.9	48± 12	1.6
Aqueous fraction[e]	100 μL	234±76	9.0	29± 5	1.0*
	200 μL	269±66	10.3	75± 18	2.7
	300 μL	142±38	5.5	162± 42	5.8
Tryptophan[f]	1 mg	27± 5	1.1*	33± 4	1.1*
	5 mg	32± 4	1.3	35± 4	1.1*

[a]Equimolar concentrations of aqueous chlorine and tryptophan (0.0 5M final concentration) were allowed to react in 0.1 M sodium phosphate buffer at ambient temperature for 24 hours. The mixture was filtered and the filtrate was concentrated 10-fold by rotary evaporation, filter-sterilized and tested for mutagenic activity in Salmonella typhimurium TA98.

[b]Mean ± standard deviations from duplicate runs using four plates/dose.

[c]Aqueous concentrates were tested as μL of sample while the dry precipitates were tested as mg sample.

[d]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[e]The concentrate was tested after 7 days storage at 4°C.

[f]Tryptophan solution (0.05 M) was concentrated by rotary evaporation and and the dry sample was filtered to serve as blank.

Table 3. Mutagenicity of the aqueous concentrate arising from the reaction of equimolar concentrations of aqueous chlorine with tryptophan using Salmonella typhimurium strain TA100[a]

		Number of revertants/plate[b]			
		TA100			
Sample	Dose/plate[c]	-S9	MR[d]	+S9	MR
Bacteria		84± 7		121± 14	
Bacteria + DMSO	25 μL	91± 15		100± 8	
Aqueous fraction	100 μL	850±241	10.1	167± 40	1.4*
	200 μL	829± 13	9.9	222±160	1.8*
	300 μL	326± 64	3.9	105± 20	0.9*
Precipitate	1 mg	152± 12	1.7	155± 10	1.5
	5 mg	155± 45	1.7	159± 31	1.6
Aqueous fraction[e]	100 μL	163± 60	1.9	135± 12	1.1*
	200 μL	393±114	4.7	273± 16	2.3
	300 μL	216± 69	2.6	121± 7	1.0*
Tryptophan[f]	1 mg	155± 16	1.7	125± 12	1.3
	5 mg	168± 21	1.8	140± 9	1.4

[a]Equimolar concentrations of aqueous chlorine and tryptophan (0.05 M final concentration) were allowed to react in 0.1 M sodium phosphate buffer at ambient temperature for 24 hours. The mixture was filtered and the filtrate was concentrated 10-fold by rotary evaporation, filter-sterilized and tested for mutagenic activity in Salmonella typhimurium TA100.

[b]Mean ± standard deviations from duplicate runs using four plates/dose.

[c]Aqueous concentrates were tested as μL of sample while the dry precipitates were tested as mg sample.

[d]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[e]The concentrate was tested after 7 days storage at 4°C.

[f]Tryptophan solution (0.05 M) was concentrated by rotary evaporation and the dry sample was filtered to serve as blank.

S-9 mix caused a reduction in the toxicity and thus allowed the actual number of revertants to be expressed.

The stability of the aqueous concentrate was checked by storing the samples in a refrigerator at 4°C for 7 days and then assaying for mutagenic activity. Some loss of mutagenic activity was observed in strain TA98 after the storage period (Table 2). In strain TA100, almost all the samples exhibited major losses in mutagenic activity. This can be seen from the mutagenicity ratio values in Table 3. The mutagenic compounds detected in these studies are mainly direct-acting mutagens which can cause both frameshift (TA98) and base-pair substitution (TA100) mutations.

Unlike the filtrate, the dark-colored precipitate and the tryptophan controls failed to exhibit mutagenic activity in both bacteria strains at all the doses tested (up to 5 mg dry sample/plate). Addition of the S-9 mix to the assay mixture did not have any observable effects on the mutagenicity of these samples (Table 2 and 3). All the positive controls, MMS, 2-AF and 2-NF were mutagenic to both tester strains at the doses tested in all the mutagenicity studies. The net number of revertants for these controls ranged from 4000-6000 revertants/plate.

Liquid-liquid extraction

In another study, the pH of the reaction mixture was adjusted with HCl and NaOH to give acidic, neutral and alkaline fractions. Each fraction was extracted separately

with hexane, methylene chloride and ethyl ether. The organic solvents were removed by rotary evaporation and the residues evaluated separately for mutagenic activity in the Ames assay. The results of these studies are presented in Tables 4 and 5. All three organic solvents extracted some colored substances from the reaction mixture, which were readily soluble in DMSO. Upon drying, the methylene chloride and ethyl ether extracts appeared dark blue to black while the hexane extracts appeared reddish in color. In some cases the amount of sample recovered was so little that it was not enough to cover the full range of doses designed to be analyzed.

The methylene chloride and ethyl ether extracts of the acidic fraction were found to be mutagenic to S. typhimurium strain TA98 in the absence of the S-9 mix while the hexane extract was not (Table 4). When the S-9 mix was added, there was a significant decrease in mutagenic activity such that mutagenicity was negligible. On a comparative basis, the methylene chloride extracts were more potent than the ethyl ether extracts at this pH for TA98. At a dose of 500 μg/plate, the methylene chloride extracts showed a relative mutagenicity value of 5.4, while it was 3.7 for the ethyl ether at 5 mg/plate (Table 4).

Toward bacteria strain TA100, only the ethyl ether extract was mutagenic at a rather high dose of 5 mg/plate (Table 5). As before, mutagenic activity was decreased in the presence of the S-9 mix. The acidic aqueous solution

Table 4. Mutagenicity of the liquid-liquid extracts of the reaction products of aqueous chlorination of tryptophan using Salmonella typhimurium strain TA98[a]

Sample	Dose/plate	Number of revertants/plate[b] TA98 -S9	MR[c]	TA98 +S9	MR
Bacteria		31± 6		35± 8	
Bacteria + DMSO	25 μL	31± 4		33± 7	
Acidic fraction					
Hexane extract	500 μg	25± 2	0.8*	25± 6	0.8*
	2 mg	55±17	1.8	38± 6	1.2*
Methylene chloride extract	500 μg	167±13	5.4	57± 2	1.7
Ether extract	500 μg	55± 8	1.8	39± 3	1.2*
	5 mg	114±14	3.7	60± 7	1.8
Aqueous fraction[d]	100 μL	T[e]		T	
	300 μL	T		T	
Neutral fraction					
Hexane extract	250 μg	72± 6	2.3	78±12	2.4
	500 μg	T		T	
	1 mg	T		T	
	5 mg	T		T	
Methylene chloride extract	250 μg	88± 9	2.8	58± 7	1.8
	500 μg	87± 8	2.8	82± 3	2.5
	1 mg	135± 7	4.4	170±19	5.2
	5 mg	T		T	
Ether extract	250 μg	247±11	8.0	103± 8	3.1
	500 μg	T		T	
	1 mg	T		T	

Table 4-continued.

Sample	Dose/plate	Number of revertants/plate[b] TA98 -S9	MR	TA98 +S9	MR
Alkaline fraction					
Hexane extract	500 μg	35± 8	1.1*	43±11	1.3*
Methylene chloride extract	500 μg	17± 3	0.5*	90±14	2.7
Ether extract	500 μg	62± 6	2.0	37± 5	1.1*
	5 mg	88±11	2.8	98±11	3.0
Aqueous fraction	100 μL	47± 4	1.5	38± 1	1.2*
	300 μL	46± 6	1.5	ND[f]	

[a]Equimolar concentrations of aqueous chlorine and tryptophan (final concentration 0.05 M) were allowed to react for 24 hours at room temperature. The reaction mixture was extracted three times with equal volumes of hexane, methylene chloride and ethyl ether to obtain neutral organic extracts. The aqueous solution was split in half and the pH was adjusted to obtain acidic (pH 1.2) and alkaline (pH 12) fractions. These two fractions were then subjected to similar solvent extractions as previously described. The solvent extracts were concentrated by rotary evaporation and the residues were weighed and taken up in DMSO for the Ames test using Salmonella typhimurium strain TA98.

[b]Means of duplicate runs of four plates/dose plus standard deviation.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]The acidic and alkaline aqueous fractions remaining after the solvent extractions were concentrated by rotary evaporation and tested for mutagenic activity.

[e]Toxic

[f]Not determined.

Table 5. Mutagenicity of the liquid-liquid extracts of the reaction products of aqueous chlorination of tryptophan using Salmonella typhimurium strain TA100[a]

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR[c]	TA100 +S9	MR
Bacteria		136± 17		130± 16	
Bacteria + DMSO	25 μL	135± 14		126± 17	
Acidic fraction					
Hexane extract	500 μg	137± 18	1.0*	161± 18	1.3
	2 mg	198± 17	1.5	170± 6	1.3
Methylene chloride extract	500 μg	223± 23	1.7	247± 21	2.0
Ether extract	500 μg	167± 31	1.2*	160± 12	1.3
	5 mg	306± 37	2.3	211± 5	1.7
Aqueous fraction[d]	100 μL	T[e]		T	
	300 μL	T		T	
Neutral fraction					
Hexane extract	250 μg	182± 11	1.3	174± 14	1.4
	500 μg	T		423±108	3.4
	1 mg	T		503± 46	4.0
	5 mg	T		T	
Methylene chloride extract	250 μg	141± 16	1.0*	99± 11	0.8*
	500 μg	269± 27	2.0	190± 15	1.5
	1 mg	493± 57	3.7	377± 29	3.0
	5 mg	T		666± 66	5.3
Ether extract	250 μg	1094±152	8.1	194± 11	1.5
	500 μg	T		1326± 77	11.0
	1 mg	T		T	

Table 5-continued.

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR	TA100 +S9	MR
Alkaline fraction					
Hexane extract	500 μg	77± 6	0.6*	180± 11	1.4
Methylene chloride extract	500 μg	118± 9	0.9*	157± 25	1.2*
Ether extract	500 μg	147± 18	1.1*	150± 10	1.2
	5 mg	373± 49	2.8	231± 17	1.8
Aqueous fraction[c]	100 μL	163± 19	1.2*	136± 12	1.1*
	300 μL	149± 8	1.1*	ND[f]	

[a]Equimolar concentrations of aqueous chlorine and tryptophan (final concentration 0.05 M) were allowed to react for 24 hours at room temperature. The reaction mixture was extracted three times with equal volumes of hexane, methylene chloride and ethyl ether to obtain neutral organic extracts. The aqueous solution was split in half and the pH was adjusted to obtain acidic (pH 1.2) and alkaline (pH 12) fractions. These two fractions were then subjected to similar solvent extractions as previously described. The solvent extracts were concentrated by rotary evaporation and the residues weighed and taken up in DMSO for the Ames test using Salmonella typhimurium strain TA100.

[b]Means of duplicate runs of four plates/dose plus standard deviation.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]The acidic and alkaline aqueous fractions remaining after solvent extractions were concentrated by rotary evaporation and tested for mutagenic activity.

[e]Toxic

[f]Not determined.

remaining after the liquid-liquid extractions was also concentrated by rotary evaporation and tested for mutagenic activity but was found to be too toxic to both tester strains of bacteria. Addition of the S-9 mix to the test system did not decrease the toxicity (Tables 4 and 5).

At neutral pH, mutagenic activity was detected in all three solvent extracts toward both strains in the presence or absence of the S-9 mix. However, the overall mutagenic activity was lower in the presence of the S-9 mix (Tables 4 and 5). Toward TA98, the ethyl ether and hexane extracts were mutagenic at 250 μg/plate, while the methylene chloride extract was mutagenic over a wide range of doses (Table 4). Higher doses of all the extracts in this fraction were highly toxic to strain TA98, and toxicity was not diminished even in the presence of the S-9 mix (Table 4). On the contrary, toxicity was reduced in strain TA100 in some but not all the levels when the S-9 mix was added (Table 5).

With regards to the alkaline or basic fraction, only the ethyl ether extract was positive in tester strain TA98 with or without the metabolic activation system (Table 4). Toward TA100, this fraction was mutagenic only in the absence of the S-9 mix (Table 5). The degree of mutagenicity in this extract increased slightly in TA98 but decreased in TA100 when the S-9 mix was present (Tables 4 and 5). The hexane and methylene chloride extracts were non-mutagenic at 500 μg/plate except for a single dose of the latter extract which was mutagenic

toward bacteria strain TA98 only in the presence of the S-9 mix (Table 4). The aqueous solution remaining after the basic compounds had been extracted was not mutagenic to either strain with or without the S-9 mix. From these studies, it was perceived that the most potent mutagens were present as neutral compounds in the reaction mixture even though some mutagenicity was present in the acidic and alkaline fractions as well. Solvent controls of methylene chloride, hexane and ether were treated the same way and the residues were dissolved in DMSO and assayed for mutagenic activity. None of the solvents was found to be mutagenic in the Ames assay.

Based on the above results, the next series of experiments using only aqueous chlorine was performed at neutral pH using a 3:1 molar ratio of chlorinating agent to tryptophan. This ratio was chosen because during food processing operations amino acid content of process water is likely to be much higher than in natural waters, and excess chlorine is used to treat and disinfect process water for re-use or discharge into the environment (Robinson et al., 1981). Reactions were conducted as previously described and extracted with the three organic solvents as before. The results of this study are presented in Tables 6 and 7. As before, mutagenic activity was detected in both tester strains in the methylene chloride and ethyl ether extracts (Tables 6 and 7). Toward TA98, the ethyl ether extract was very potent and exhibited a dose related increase in mutagenic activity

Table 6. Mutagenicity of the liquid-liquid extracts of the reaction products arising from the chlorination of tryptophan using Salmonella typhimurium strain TA98[a]

		Number of revertants/plate[b]			
		TA98			
Sample	Dose/plate	-S9	MR[c]	+S9	MR
Bacteria		17± 3		22± 4	
Bacteria + DMSO	25 μL	18± 4		22± 5	
Hexane extract	500 μg	21± 8	1.2*	32± 6	1.5
	1 mg	ND[d]		ND	
	5 mg	T[e]		T	
Methylene chloride extract	500 μg	26± 9	1.4*	41± 8	1.9
	1 mg	31± 3	1.7	42± 9	1.9
	5 mg	58±10	3.2	42±10	1.9
Ether extract	500 μg	130± 9	7.2	53± 2	2.4
	1 mg	276±22	15.3	98± 7	4.5
	2.5 mg	293±37	16.3	111± 5	5.0
	5 mg	T		T	

[a]A 3:1 molar ratio of aqueous chlorine (0.1 M) and tryptophan (0.03 M) in phosphate buffer (pH 7) were allowed to react at room temperature for 24 hours. The reaction mixture was extracted three times each with equal volumes of hexane, methylene chloride and ethyl ether. The organic solvents were removed by rotary evaporation and the residues were weighed and dissolved in DMSO and used for the Ames assay in Salmonella typhimurium strain TA98.

[b]Results of duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denoted not significantly different from control samples (P=0.05).

[d]Not determined.

[e]Toxic.

Table 7. Mutagenicity of the liquid-liquid extracts of the reaction products arising from the chlorination of tryptophan using Salmonella typhimurium strain TA100[a]

		Number of revertants/plate[b]			
		TA100			
Sample	Dose/plate	-S9	MR[c]	+S9	MR
Bacteria		133± 9		128±12	
Bacteria + DMSO	25 μL	133± 5		104±12	
Hexane extract	500 μg	138± 15	1.0*	84±16	0.8*
	1 mg	ND[d]		ND	
	5 mg	T[e]		T	
Methylene chloride extract	500 μg	125± 9	0.9*	170±35	1.6
	1 mg	246± 14	1.8	195± 9	1.9
	5 mg	550± 82	4.1	281±22	2.7
Ether extract	500 μg	1108± 54	8.3	303±36	2.9
	1 mg	1570±128	11.8	485±54	4.7
	2.5 mg	1833±112	13.8	899±55	8.6
	5 mg	T		T	

[a]A 3:1 molar ratio of aqueous chlorine (0.1 M) and tryptophan (0.03 M) in phosphate buffer (pH 7) were allowed to react at room temperature for 24 hours. The reaction mixture was extracted three times each with equal volumes of hexane, methylene chloride and ethyl ether. The organic solvents were removed by rotary evaporation and the residues were weighed and dissolved in DMSO and used for the Ames assay in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denoted not significantly different from control samples (P=0.05).

[d]Not determined.

[e]Toxic.

until at some higher dose where toxicity was encountered (Table 6). Again, the addition of the S-9 mix to the test system led to a more than 2-fold decrease in mutagenic potency of this extract (Table 6). The methylene chloride extract was only weakly mutagenic in TA98 with the activity being abolished in the presence of the S-9 mix (Table 6). Both the ethyl ether and methylene chloride extracts showed dose related increases in mutagenic activity in TA100 with the potencies decreasing in the presence of the S-9 mix (Table 7). The hexane extracts were either nonmutagenic overall, or were toxic to the bacteria at the doses tested (Tables 6 and 7).

On a comparative basis, the potency of the 3:1 extracts were much lower than the 1:1 extracts on a dose to dose basis (Tables 4 and 6). For example at the 3:1 ratio, a linear dose-response mutagenic activity curve was obtained for the ethyl ether extracts at doses of 500 μg up to 2.5 mg/plate (Tables 6 and 7) while the same does not hold true for the 1:1 extracts. For the 1:1 ratio, a minimum dose of 250 μg/plate gave results comparable to a dose of 2.5 mg/plate in the 3:1 ratio. The overall reduction in the mutagenic activity was less and was probably caused by the formation of less toxic compounds at the 3:1 ratio, or due to the formation of products which are not extractable with the organic solvents used. Results of the liquid-liquid extractions also indicate that ethyl ether is the most suitable organic solvent for the

extraction of the relatively non-polar reaction mixtures of aqueous chlorination of tryptophan.

Mutagenicity of the Reaction Products of Aqueous Chlorine or Chlorine Dioxide with L-Tryptophan

The next series of experiments was conducted to compare the mutagenicities of the reaction products of aqueous chlorine or chlorine dioxide with tryptophan. Lower tryptophan concentrations (7 and 5 mM final concentrations) were selected such that they would be representative of those amounts likely to be encountered in food processing operations. The ratios of aqueous chlorine or chlorine dioxide to amino acid, 7:1, 3:1 and 1:1, were also chosen to cover the range of concentrations likely to be encountered in the real world situation. Samples were prepared as depicted in Fig. 6 and the concentrates evaluated for mutagenic activity.

Liquid-liquid extraction

Aqueous chlorine. The results of the liquid-liquid extraction of the aqueous chlorine-tryptophan reaction products using ethyl ether as the extracting solvent are presented in Tables 8 and 9. The reaction products of aqueous chlorine with tryptophan were mutagenic to TA98 without metabolic activation at the 1:1 ratio. Mutagenic activity was abolished at the 3:1 ratio and increased dramatically at the 7:1 ratio (Table 8). Higher doses of the 3:1 and 7:1 ratios were toxic to the

Table 8. Mutagenicity of the ethyl ether extracts arising from the aqueous chlorination of tryptophan using Salmonella typhimurium strain TA98[a]

		Number of revertants/plate[b]			
		TA98			
Sample	Dose/plate	-S9	MR[c]	+S9	MR
Bacteria		20± 6		29± 3	
Bacteria + DMSO	25 μL	21± 3		28± 4	
1:1 Extract					
	1 mg	42± 9	2.0	36± 5	1.3
	2 mg	58± 6	2.8	47±10	1.7
	5 mg	68±12	3.2	42±11	1.5
3:1 Extract					
	250 μg	29± 5	1.4	36± 5	1.3
	1 mg	38± 2	1.8	47± 5	1.7
	5 mg	T[d]		T	
7:1 Extract					
	500 μg	174±18	8.3	68± 7	2.4
	1 mg	237±16	11.3	92± 2	3.3
	5 mg	T		285± 8	10.2

[a]Three molar ratios of aqueous chlorine to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each mixture were extracted separately with ethyl ether and concentrated for mutagenicity assessment in Salmonella typhimurium strain TA98.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

Table 9. Mutagenicity of the ethyl ether extracts arising from the aqueous chlorination of tryptophan using Salmonella typhimurium strain TA100[a]

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR[c]	+S9	MR
Bacteria		123± 9		120± 9	
Bacteria + DMSO	25 μL	124± 9		124± 5	
1:1 Extract					
	1 mg	106± 9	0.9*	126± 27	1.0*
	2 mg	156±20	1.3	151± 9	1.2
	5 mg	134±10	1.1*	217± 23	1.8
3:1 Extract					
	1 mg	179±37	1.4	143± 7	1.2
	2 mg	106±21	0.9*	146± 13	1.2
	5 mg	T[d]		T	
7:1 Extract					
	1 mg	667±51	5.4	407± 32	3.3
	2 mg	706±71	5.7	644± 59	5.2
	5 mg	T		1203±123	9.7

[a]Three molar ratios of aqueous chlorine to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each mixture were extracted separately with ethyl ether and concentrated for mutagenicity assessment in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

bacteria, while those of the 1:1 ratio were not. Addition of the S-9 mix to the test system led to a decline in the mutagenic activity of all the treatments and decreased the toxicity in the 7:1 ratio. However, the addition of S-9 mix did not decrease the toxicity observed in the 3:1 ratio (Table 8).

Toward bacteria strain TA100, only the 7:1 ratio was mutagenic with mutagenic activity decreasing in the presence of the S-9 mix (Table 9). As before, higher concentrations of the 7:1 and 3:1 ratios were toxic to the bacteria. Toxicity in the 7:1 and not the 3:1 ratio decreased in the presence of the S-9 mix and the actual number of revertants was expressed. The results in Tables 8 and 9 indicate that the ethyl ether extractable reaction products are mainly frameshift mutagens with greater potency towards TA98 and are also capable of causing base-pair substitution mutations in TA100.

Chlorine dioxide. A similar pattern was observed for the reaction products of chlorine dioxide with tryptophan. The reaction products for the three ratios showed mutagenic activity in tester bacteria TA98 in the absence of the S-9 mix (Table 10). However, the overall mutagenic activity decreased slightly from the 1:1 to the 3:1 ratio and increased again in the 7:1 ratio for this bacteria strain. Addition of the S-9 mix led to a decline in all the ratios except at the 1 mg/plate dose of the 3:1 ratio (Table 10). In the case of

Table 10. Mutagenicity of the ethyl ether extracts arising from the reaction of aqueous chlorine dioxide with tryptophan using Salmonella typhimurium strain TA98[a]

		Number of revertants/plate[b]			
		TA98			
Sample	Dose/plate	-S9	MR[c]	+S9	MR
Bacteria		25± 3		23± 4	
Bacteria + DMSO	25 μL	21± 3		22± 7	
1:1 Extract					
	1 mg	63±10	3.0	30± 3	1.4*
	2 mg	111± 6	5.3	64± 9	3.0
	5 mg	168±14	8.0	152±19	7.2
3:1 Extract					
	1 mg	34±10	1.6	112± 4	5.3
	2 mg	126±37	6.0	95±11	4.5
	5 mg	112± 8	5.3	101±10	4.8
7:1 Extract					
	1 mg	107±16	5.1	57± 8	2.7
	2 mg	163±25	7.8	98±14	4.7
	5 mg	250±33	11.9	141± 5	6.7

[a]Three molar ratios of aqueous chlorine dioxide to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each mixture were extracted separately with ethyl ether and concentrated for mutagenicity assessment in Salmonella typhimurium strain TA98.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

S. typhimurium strain TA100, increasing the molar ratio of chlorine dioxide from 1 to 7 led to an overall increase in the mutagenic activity, especially at the highest doses tested (Table 11). Again the addition of the rat hepatic S-9 mix led to a decline in the potency of some but not all the extracts. Also, mutagenic activity was not abolished at all the doses tested (Table 11). Looking at the ratio where both produced mutagens, it is clear that aqueous chlorine reaction products were more potent than those of aqueous chlorine dioxide (Tables 8 and 10). These results indicate that both chlorine dioxide and aqueous chlorine are capable of producing potent frameshift mutagens when reacted with tryptophan. No toxicity was observed in the ethyl ether extracts of chlorine dioxide-tryptophan reaction mixtures even at very high doses of up to 5 mg/plate (Tables 10 and 11). However, similar doses of the aqueous chlorine reaction products were mostly toxic especially at the higher chlorine to tryptophan ratios (Tables 8 and 9). Also ethyl ether is a suitable organic solvent capable of extracting some but not all of the mutagens present in the aqueous reaction mixtures.

Amberlite XAD adsorption

Distribution of radioactivity. Preliminary experiments were conducted to find the best method for concentrating the chlorination reaction mixtures using Amberlite XAD-2 and XAD-8 either separately in different columns or in combination in one column. The results of this study indicated that the

Table 11. Mutagenicity of the ethyl ether extracts arising from the reaction of aqueous chlorine dioxide with tryptophan using Salmonella typhimurium strain TA100[a]

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR[c]	+S9	MR
Bacteria		135±11		123± 3	
Bacteria + DMSO	25 μL	140±14		118± 8	
1:1 Extract					
	1 mg	182±13	1.3	159±20	1.3
	2 mg	371±42	2.7	216±12	1.8
	5 mg	599±17	4.3	497±90	4.2
3:1 Extract					
	1 mg	176±21	1.3	163±18	1.4
	2 mg	342±19	2.4	257±23	2.1
	5 mg	659±56	4.7	567±55	4.8
7:1 Extract					
	1 mg	252±13	1.8	244±32	2.1
	2 mg	449±64	3.2	330± 4	2.8
	5 mg	844±67	6.0	605±57	6.7

[a]Three molar ratios of aqueous chlorine dioxide to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each mixture were extracted separately with ethyl ether and concentrated for mutagenicity assessment in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls.

recovery of the reaction products was irrespective of whether XAD-2 resins were placed on top of the XAD-8 resins or vice versa (Table 12). In both cases, using the two resins in one column provided better recoveries than using XAD-2 alone (Table 12). When XAD-8 was placed on top of XAD-2, the recoveries were slightly higher (48% against 46%), but again, the differences were not statistically significant (Table 12). Data on the acidification of the reaction mixture prior to Amberlite XAD adsorption are presented in Table 13. Maximum recovery of the reaction products was obtained at pH 2.5 (60% recovery) than at pH 6.0 (45% recovery) (Table 13). Also, at any given pH, product recovery was independent of whether aqueous chlorine or chlorine dioxide was used (Table 13).

The amount of radioactivity recovered was less for reaction mixtures with excess chlorine or chlorine dioxide (7:1 molar ratio) than those at the 1:1 ratio. However, the eluates in the aqueous chlorine and chlorine dioxide reaction mixtures at the 7:1 ratio were not significantly different from each other (Table 14).

Mutagenicity of the Amberlite XAD eluates

Control solutions. Procedural blanks containing aqueous chlorine, chlorine dioxide or tryptophan alone in 0.1 M sodium phosphate buffer were mixed for 24 hours as described for the actual samples. After reaction, all the samples including the tryptophan solution were dechlorinated with sodium sulfite. The control blanks were then concentrated by resin adsorption,

Table 12. Percent distribution of radioactivity after resin adsorption in the reaction of aqueous chlorine with tryptophan[a]

Sample fraction	XAD-8/2[b]	XAD-2/8[b]	XAD-2
Filtrate[c]	100	100	100
Column effluent	48.9±2.7	43.3±0.5	54.2±1.6
Column eluate[d]	48.0±0.6[x]	45.5±1.7[x]	37.5±0.5[y]
Loss[e]	3.1±0.1	11.2±0.3	8.3±0.1

[a]Aqueous chlorine was allowed to react with a radiolabeled tryptophan solution (1:1 molar ratio) for 4 hours. The reaction mixture was filtered and concentrated by resin adsorption.

[b]XAD-8/2 is equivalent to placing XAD-8 on top of XAD-2 (1:1 mixture) and vice versa.

[c]After filtration, there was about 20% loss of radioactivity. Therefore the filtrate was assumed to contain 100% product prior to resin adsorption.

[d]Means with the same letter in the same row are not significantly different from each other ($P<0.05$).

[e]The amount of radioactivity unaccounted for was assumed to be irreversibly linked to the resins or lost through volatilization.

Table 13. Recovery of radioactivity after resin adsorption in the reaction of aqueous chlorine and chlorine dioxide with tryptophan[a]

Sample fraction	pH 6.0		pH 2.5	
	Tryptophan + HOCl	Tryptophan + ClO_2	Tryptophan + HOCl	Tryptophan + ClO_2
Filtrate[b]	100	100	100	100
Column effluent	48.9±2.7	44.4±2.3	35.7±2.9	36.1±0.5
Column eluate[c]	48.0±0.6^{x}	44.0±1.4^{x}	56.5±3.1^{y}	60.6±1.8^{y}
Loss[d]	3.1±0.1	11.6±0.2	7.8±0.2	3.3±0.2

[a]Aqueous chlorine or chlorine dioxide was allowed to react with a radiolabeled tryptophan solution for 4 hours at room temperature at pH 6.0. The reaction was filtered and the mixture divided into two portions. The pH of one-half was adjusted to 2.5 with 85% phosphoric acid and both fractions concentrated separately by resin adsorption with Amberlite XAD-8/2 (1:1 mixture). The adsorbed substances were eluted successively with acetone, methanol and ethyl ether. The eluates were concentrated by rotary evaporation, adjusted to a fixed volume by the addition of chlorine demand-free water, and aliquots were checked for radioactivity.

[b]After filtration there was about 20% loss of radioactivity. Therefore the filtrate was considered to be 100%.

[c]Means with same letter in the same row are not significantly different from each other ($P<0.05$).

[d]The amount of radioactivity unaccounted for was assumed to be irreversibly linked to the resins or lost through volatilization.

Table 14. Percent distribution of radioactivity after resin adsorption in the reaction of aqueous chlorine or chlorine dioxide with tryptophan[a]

Sample fraction	Tryptophan + buffer	Tryptophan + HOCl	Tryptophan + ClO_2
Filtrate[b]	100	100	100
Column effluent	35.6±2.1	48.6±2.0	41.2±0.8
Column eluate[c]	$58.4±1.1^{x}$	$49.5±0.7^{y}$	$48.6±1.2^{y}$
Loss[d]	6.0±0.2	3.7±0.1	10.2±0.3

[a]Aqueous chlorine, chlorine dioxide or buffer were allowed to react with a radiolabelled tryptophan solution (7:1 molar ratio) at room temperature for 24. Any excess chlorine or chlorine dioxide remaining after reaction was removed by addition of sodium sulfite and the solutions were filtered and concentrated by resin adsorption. The adsorbed substances were successively eluted with acetone, methanol and ethyl ether. The eluates were concentrated by rotary evaporation and aliquots checked for radioactivity.

[b]After filtration, the filtrate was assumed to be 100% starting material. There was about 20% loss of radioactivity in the precipitate or as volatiles.

[c]Means with the same letter in the same row are not significantly different from each other ($P<0.05$).

[d]The amount of radioactivity that was not accounted for was assumed to be trapped in the resins or lost through volatilization.

eluted with acetone, methanol and ethyl ether, respectively, and assayed for mutagenic activity as previously described. The results indicated that none of the procedural blanks was mutagenic in either strain of bacteria at the doses tested (Table 15).

Aqueous chlorine. Sample preparation for the above reactions are summarized in Fig. 6. Mutagenic activity was detected in all the Amberlite XAD acetone eluates in strain TA98 at the three ratios of aqueous chlorine to the amino acid in the absence of the S-9 mix (Table 16). In all cases, the acetone eluates contained the most potent mutagens as compared to the methanol eluates at the three ratios. A dose dependent increase in mutagenic activity was observed in all these eluates until at some higher dose when toxicity was encountered. Again, the degree of mutagenicity increased with increasing chlorine concentration. For example at a test dose of 100 μL/plate, the relative mutagenicity ratios were 4.3, 6.9 and 15.7, respectively, for the 1:1, 3:1 and 7:1 ratios of aqueous chlorine:tryptophan extracts (Table 16).

The methanol eluates were mutagenic at the 1:1 and 3:1 ratios but not at the 7:1 ratio toward bacteria strain TA98. The 3:1 ethyl ether eluate was only weakly mutagenic to this strain of bacteria. No ethyl ether eluates were present in the 1:1 and 7:1 samples because examination of the column for color indicated that all the adsorbed substances had been removed by the acetone and methanol. Also, subsequent elution

Table 15. Mutagenicity of Amberlite XAD eluates arising from control aqueous chlorine, chlorine dioxide and tryptophan solutions[a]

Sample	Dose/plate	Number of revertants/plate[b] TA98 -S9	TA98 MR[c]	TA100 -S9	TA100 MR
Bacteria		35± 2		92±15	
Bacteria + DMSO	25 µL	36± 4		84± 7	
Aqueous chlorine					
Acetone eluate	100 µL	40± 2	1.1	94±17	1.0*
	300 µL	38± 3	1.1*	93± 6	1.0*
Methanol eluate	250 µg	37± 3	1.0*	90±11	1.1*
	1 mg	37± 3	1.0*	103±13	1.2
	2 mg	38± 1	1.1*	98±10	1.2*
Ether eluate	500 µg	36± 4	1.0*	84±12	1.0*
Chlorine dioxide					
Acetone eluate	100 µL	43± 3	1.2	84± 6	0.9*
	300 µL	40± 6	1.1*	90±18	1.0*
Methanol eluate	250 µL	38± 2	1.1*	89± 3	1.1*
	1 mg	35± 3	1.0*	89± 7	1.1*
	5 mg	38± 4	1.1*	99±11	1.2*
Ether eluate	500 µL	26± 1	0.7*	84± 5	1.0*
Tryptophan					
Acetone eluate	100 µL	37± 3	1.1*	83±11	0.9*
	300 µL	38± 2	1.1*	91± 5	1.0*
Methanol eluate	250 µg	35± 2	1.0*	89± 8	1.1*
	1 mg	36± 3	1.0*	103±16	1.2*
	5 mg	38± 2	1.1*	91± 5	1.1*
Ether eluate	500 µg	36± 2	1.0*	98±13	1.2*

Table 15-continued.

[a]Aqueous chlorine, chlorine dioxide or tryptophan alone were mixed in phosphate buffer for 24 hours. After reaction any available chlorine present in the aqueous chlorine and chlorine dioxide solutions were removed by the addition of sodium sulfite. The solutions from each mixture were then concentrated by resin adsorption and eluted separately with acetone, methanol and ethyl ether. The eluates were concentrated separately by rotary evaporation and aliquots assayed for mutagenic activity in Salmonella typhimurium strains TA98 and TA100.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

Table 16. Mutagenicity of Amberlite XAD eluates arising from the reaction of aqueous chlorine with tryptophan using Salmonella typhimurium strain TA98[a]

		Number of revertants/plate[b]			
		TA98			
Sample	Dose/plate	-S9	MR[c]	+S9	MR
Bacteria		20± 6		29± 3	
Bacteria + DMSO	25 µL	21± 3		28± 4	
1:1 Acetone eluate					
	100 µL	86±25	4.3	52±14	1.8
	200 µL	196± 7	9.8	73± 8	2.5
	300 µL	T[d]		T	
1:1 Methanol eluate					
	1 mg	38± 6	1.8	44± 7	1.6
	2 mg	44± 2	2.1	36±12	1.3
	5 mg	85±12	4.0	56±11	2.0
3:1 Acetone eluate					
	50 µL	107±20	5.4	39± 6	1.3
	100 µL	138± 9	6.9	48± 4	1.7
	200 µL	T		T	
3:1 Methanol eluate					
	1 mg	35± 2	1.7	45± 2	1.6
	2 mg	60± 8	2.9	50± 8	1.8
3:1 Ether eluate					
	500 µg	33± 7	1.6	39± 5	1.4
	1 mg	42± 3	2.0	50± 8	1.8
7:1 Acetone eluate					
	50 µL	199±13	10.0	114± 8	3.9
	100 µL	313±30	15.7	126±28	4.3
	200 µL	T		T	
7:1 Acetone eluate precipitate[e]					
	500 µg	71± 8	3.4	46± 6	1.6
	2 mg	137±21	6.5	95±10	3.4
	5 mg	T		T	
7:1 Methanol eluate					
	500 µg	29± 4	1.4	50± 5	1.8
	1 mg	30± 6	1.4	55± 8	2.0

Table 16-continued.

[a]Three molar ratios of aqueous chlorine to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each reaction were concentrated by XAD adsorption and then eluted separately with acetone, methanol and ethyl ether. The eluates were concentrated by rotary evaporation and assayed for mutagenic activity in Salmonella typhimurium strain TA98.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

[e]After concentrating the acetone eluate from this ratio, some precipitate was obtained which was dissolved in DMSO and tested for mutagenic activity.

with ethyl ether provided negative or negligible yields which could not be assayed.

Addition of the S-9 mix to the test system led to a decline in the mutagenic activity in all but one of the eluates. More than a 2-fold decrease in mutagenic activity was observed in the highly mutagenic acetone eluates at all the levels and ratios tested. However, in the case of the 7:1 methanol eluate, weak mutagenic activity was detected only in the presence of the S-9 mix (Table 16). At the 7:1 ratio, a precipitate was formed upon removing the acetone by rotary evaporation. The precipitate tested positive in TA98 and the potency decreased in the presence of the S-9 mix (Table 16).

Toward S. typhimurium TA100, only the 1:1 and 7:1 acetone eluates and 7:1 precipitate were mutagenic at the levels tested when the S-9 mix was absent (Table 17). The 7:1 acetone eluate was highly mutagenic at a test dose of 50 μL/plate with a mutagenicity ratio of 17.9 Increasing the doses above 50 μL/plate led to toxicity in the bacteria. The 3:1 acetone eluate was not mutagenic but toxic to TA100 at the highest dose level tested. This lends further credence to the observation that the mutagenic compounds are mainly frameshift mutagens. Addition of the S-9 mix to these compounds led to a decrease in the potency of the 1:1 and 7:1 ratios but increased potency of the precipitate and the 3:1 acetone eluate. The ethyl ether and methanol eluates were non-

mutagenic in TA100 with or without the rat hepatic enzyme metabolizing system (Table 17).

Chlorine dioxide. With chlorine dioxide, the acetone concentrates of the XAD eluates were all mutagenic to TA98 in the absence of the S-9 mix. Again mutagenic activity decreased at the 3:1 chlorine dioxide:tryptophan ratio and increased further at the 7:1 ratio. The mutagenicity ratios for a test dose of 200 μL/plate were 7.1, 3.9 and 8.5, respectively for the 1:1, 3:1 and 7:1 ratios of chlorine dioxide:tryptophan reaction products (Table 18). In the absence of the S-9 mix, the ethyl ether and methanol eluates were weakly mutagenic or non-mutagenic. Unlike the 7:1 aqueous chlorine eluate, no precipitate was formed in the 7:1 chlorine dioxide reaction. In the presence of the S-9 mix, a decrease in mutagenic activity was observed in all the acetone eluates of the 3 ratios even though mutagenic activity was not abolished. Also, the 1:1 ethyl ether and the 3:1 methanol eluates became mutagenic on addition of the S-9 mix, which suggests that these solvents were extracting some mutagenic compounds from the column which are not direct-acting but require metabolic activation (Table 18). The only eluate which showed any toxicity toward TA98 was the 1:1 acetone extract and toxicity was not diminished in the presence of the S-9 mix.

Toward strain TA100, the acetone eluates of all 3 ratios of the chlorine dioxide-tryptophan reaction products increased

Table 17. Mutagenicity of Amberlite XAD eluates arising from the reaction of aqueous chlorine with tryptophan using Salmonella typhimurium strain TA100[a]

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR[c]	+S9	MR
Bacteria		123± 9		120± 9	
Bacteria + DMSO	25 µL	124± 9		124± 5	
1:1 Acetone eluate					
	100 µL	225± 14	1.8	120±16	1.0*
	200 µL	299± 56	2.4	254± 4	2.1
	300 µL	T[d]		T	
1:1 Methanol eluate					
	500 µg	141± 8	1.1	163±21	1.3
	1 mg	152± 16	1.2	163± 9	1.3
	2 mg	129± 19	1.0*	173± 3	1.4
3:1 Acetone eluate					
	100 µL	188± 30	1.5	85±12	0.7*
	200 µL	214± 30	1.7	310±29	2.6
	300 µL	T		T	
3:1 Methanol eluate					
	1 mg	134± 3	1.1*	135± 4	1.1
	2 mg	156± 11	1.3	153±15	1.2
3:1 Ether eluate					
	500 µg	135± 14	1.1*	151± 4	1.2
	1 mg	117± 8	0.9*	161±19	1.3
7:1 Acetone eluate					
	25 µL	1556±151	12.7	758±53	6.3
	50 µL	2205± 55	17.9	989±77	8.2
	100 µL	T		T	
7:1 Acetone eluate precipitate[e]	2 mg	539± 34	4.3	459±43	3.7
	5 mg	491± 62	4.0	717±60	6.0
7:1 Methanol eluate					
	500 µg	124± 11	1.0*	151± 7	1.2
	1 mg	158± 7	1.3	177± 8	1.4

Table 17-continued.

[a]Three molar ratios of aqueous chlorine to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each reaction were concentrated by XAD adsorption and eluted separately with acetone, methanol and ethyl ether. The eluates were concentrated by rotary evaporation and assayed for mutagenic activity in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

[e]After concentrating the acetone eluate from this ratio, some precipitate was obtained which was dissolved in DMSO and tested for mutagenic activity.

Table 18. Mutagenicity of Amberlite XAD eluates arising from the reaction of aqueous chlorine dioxide with tryptophan using Salmonella typhimurium strain TA98[a]

Sample	Dose/plate	Number of revertants/plate[b] TA98 -S9	MR[c]	+S9	MR
Bacteria		25± 3		23± 3	
Bacteria + DMSO	25 μL	21± 3		20± 7	
1:1 Acetone eluate					
	100 μL	121±22	4.8	70± 6	3.0
	200 μL	177±33	7.1	62±16	2.7
	300 μL	T[d]		T	
1:1 Methanol eluate					
	1 mg	41± 3	2.0	38± 5	1.9
	2 mg	35±14	1.7	40± 6	2.0
1:1 Ether eluate					
	1 mg	18± 4	0.9*	29± 5	1.4*
	2 mg	16± 4	0.8*	43±11	2.2
3:1 Acetone eluate					
	50 μL	74± 3	3.0	43± 4	1.9
	100 μL	112±13	4.5	58± 4	2.5
	200 μL	97±27	3.9	62± 8	2.7
3:1 Methanol eluate					
	1 mg	26±11	1.2*	46± 6	2.3
	2 mg	31±15	1.5*	56±11	2.8
	5 mg	36± 6	1.7	110± 3	5.5
7:1 Acetone eluate					
	100 μL	178±32	7.1	98± 6	4.3
	200 μL	213±15	8.5	93±16	4.0
	300 μL	161±26	6.4	118±12	5.1
7:1 Methanol eluate					
	1 mg	25± 4	1.2*	32± 5	1.6
	2 mg	30± 8	1.4*	34± 7	1.7
	5 mg	47± 7	2.2	28± 5	1.4*

Table 18-continued.

[a]Three molar ratios of aqueous chlorine dioxide to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each reaction were concentrated by XAD adsorption and eluted separately with acetone, methanol and ethyl ether. The eluates were concentrated by rotary evaporation and assayed for mutagenic activity in Salmonella typhimurium strain TA98.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

with increasing chlorine dioxide concentrations in the absence of the S-9 mix. Toxicity was observed in the 1:1 and 3:1 acetone eluates but not in the 7:1 eluates. When the S-9 mix was present, there was an overall increase in the mutagenic activity in most of these samples as shown by their relative mutagenicity ratios (Table 19). The methanol and ethyl ether eluates were non-mutagenic either in the presence or absence of the S-9 mix. Again by comparing the relative mutagenicity ratios, it appears that the mutagens are mainly frameshift mutagens even though they are also capable of giving rise to base-pair substitution mutations.

Fractionation of the Reaction Products of Aqueous Chlorine or Chlorine Dioxide and Tryptophan

Thin layer chromatographic analyses of the chlorination reaction products

A schematic representation of sample preparation procedures for the TLC analyses is presented in Fig. 7. Different solvent systems and solvent combinations were evaluated for the separation of the acetone eluates of the Amberlite XAD-2/8 concentrates. The solvents included chloroform, acetone, methanol, benzene, propanol, acetic acid and methylene chloride. A solvent system containing hexane, dichloromethane, ethyl ether, methanol and acetic acid was found to be suitable for developing the plates. With the 7:1 aqueous chlorine:tryptophan extracts, five separate identifiable fluorescent bands were obtained under a long

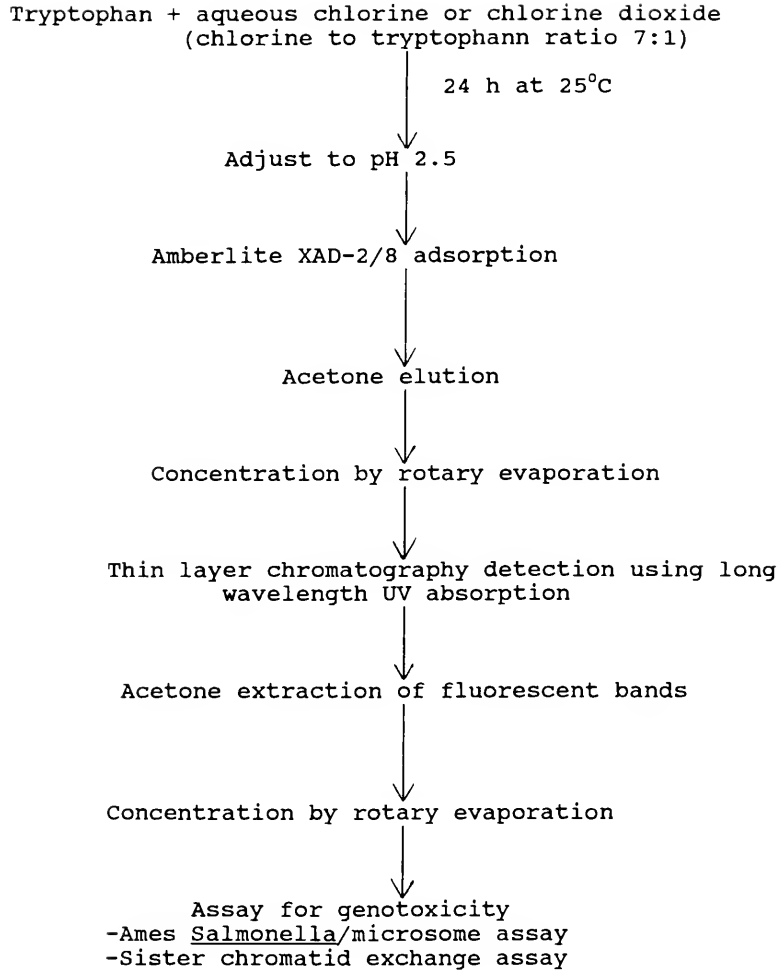

Figure 7. Schematic presentation of sample preparation for thin layer chromatography and genotoxicity assessment

Table 19. Mutagenicity of Amberlite XAD eluates arising from the reaction of aqueous chlorine dioxide with tryptophan using Salmonella typhimurium strain TA100[a]

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR[c]	+S9	MR
Bacteria		135±11		123± 3	
Bacteria + DMSO	25 μL	140±10		118± 8	
1:1 Acetone eluate					
	100 μL	332±18	2.7	240±14	2.0
	200 μL	391±29	2.9	400±16	3.3
	300 μL	T[d]		T	
1:1 Methanol eluate					
	1 mg	152±12	1.1*	142±17	1.2
	2 mg	152±12	1.1*	145± 8	1.2
1:1 Ether eluate					
	1 mg	170±27	1.2*	109± 5	0.9*
	2 mg	139± 6	1.0*	103±12	0.9*
3:1 Acetone eluate					
	100 μL	463±53	3.4	496±28	4.0
	200 μL	529±44	3.9	512±71	4.2
	300 μL	T		T	
3:1 Methanol eluate					
	2 mg	148±12	1.1*	122± 4	1.0*
	5 mg	158±13	1.1*	122±11	1.0*
7:1 Acetone eluate					
	50 μL	371±67	2.7	131±37	1.1*
	200 μL	373±23	2.8	512±37	4.2
	300 μL	656±69	4.9	1036±22	8.4
7:1 Methanol eluate					
	2 mg	160±28	1.1*	113±37	1.0*
	5 mg	167±14	1.2	131±11	1.1*

Table 19-continued.

[a]Three molar ratios of aqueous chlorine dioxide to tryptophan (1:1, 3:1 and 7:1) were allowed to react at room temperature for 24 hours. The reaction products from each reaction were concentrated by XAD adsorption and eluted separately with acetone, methanol and ethyl ether. The eluates were concentrated by rotary evaporation and assayed for mutagenic activity in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means plus standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

wavelength UV lamp on the TLC plates developed with a hexane:ethyl ether:acetic acid (50:50:1, v/v/v) solvent system. These bands were designated band # 1-5. Band #1 with an R_f value 0 retained some highly polar colored products with relatively no mobility on the TLC plates. Band #2 with an R_f value of 0.08 moved very little from the origin and was composed of mainly polar compounds. These two TLC bands represented the most polar compounds in the reaction mixture and due to their greater affinity for the polar stationary phase, their movements were limited. Bands #3 and 4 with R_f values of 0.22 and 0.38, respectively, represented compounds of intermediate polarity and were much better resolved than the previous bands. The last band, #5, with an R_f value of 0.54 was the least polar of all the five bands. Bands #1, 3 and 4 fluoresced green under the long wavelength UV lamp at 366 nm while bands #2 and 5 fluoresced blue. The fluorescent intensities of bands 3 and 4 were similar while band 5 fluoresced much more brightly than band #2.

Due to the high mutagenic activity of band #5, it was further fractionated on another TLC plate using a solvent system containing hexane, methylene chloride and acetic acid (50:50:2, v/v/v). The products from the second dimension TLC fractions were then viewed under the UV lamp to determine the presence of fluorescent bands. Three major fluorescent bands could be visualized from the TLC plates which showed both blue and green fluorescence. The blue fluorescent compounds were

designated band 5c with an R_f value of 0.31 and band 5h with an R_f value of 0.88, respectively. The green fluorescent band 5e had an R_f value of 0.62.

With chlorine dioxide only 4 fluorescent bands could be visualized under a UV lamp at 366 nm from the TLC plates developed with the hexane:ethyl ether: methanol:acetic acid (63:30:7:1, v/v/v/v) solvent system. The fractions also showed fluorescence in the short wavelength UV range (254 nm) but of much lower intensity. The bands were designated A, B, C and D with corresponding R_f values of 0, 0.13, 0.31 and 0.44. The reaction products in these fluorescent bands appeared to be more polar than those from aqueous chlorine-treated samples due to their relatively low mobility on the TLC plates. Bands A and B fluoresced green while C and D fluoresced blue with D being the most intense.

For both aqueous chlorine and chlorine dioxide reaction products, there was some loss of fluorescence when the plates were left in the open at ambient temperature for more than 2 hours and about 90% of the fluorescence disappeared after about 48 hours. However, fluorescence was maintained for an extended period of time when the bands were extracted with and stored in acetone. Judging from the R_f values of the fluorescent compounds, none of the byproducts of the reactions between tryptophan and aqueous chlorine or chlorine dioxide appeared similar.

Product distribution in the TLC fractions. Aliquots of the radioactive samples (Fig. 7) were spotted on TLC plates and developed as previously described. The results indicated that most of the radioactivity was in the polar fractions for both aqueous chlorine and chlorine dioxide reaction products (Table 20). Due to their high affinity for the silica gel, these polar compounds had little or no movement from the origin. The fluorescent compounds in the aqueous chlorine reaction products accounted for a total of 87.3% of the applied radioactivity with most of the product (64%) being in the origin. The chlorine dioxide reaction products accounted for about 95% of the applied sample and again, most of the radioactivity was in the polar fractions Bands A and B (92%) (Table 20).

The highly mutagenic fraction (Band #5) in the aqueous chlorine reaction was about 1.4% of the total radioactivity, while in the case of chlorine dioxide, the mutagenic fraction (Band #C) accounted for only 2% of the total radioactivity (Table 20).

Mutagenic activity of the TLC extracts of the reaction products of aqueous chlorine and chlorine dioxide with tryptophan

The fluorescent bands were scraped from the TLC plates and the compounds from each band were extracted separately with acetone. The acetone extracts were then filtered to remove the silica and concentrated by rotary evaporation under vacuum. Upon drying, some brown-colored substances similar

Table 20. Distribution of radioactivity in the TLC subfractions of the reaction products of aqueous chlorine or chlorine dioxide with tryptophan[a]

Aqueous chlorine	(% Radio-activity[b])	Chlorine dioxide	(% Radio-activity)
Band #		Band #	
1 (R_f=0)	64.1	A (R_f=0)	15.0
2 (R_f=0.08)	11.0	B (R_f=0.13)	77.0
3 (R_f=0.22)	8.7	C (R_f=0.31)	2.0
4 (R_f=0.38)	1.8	D (R_f=0.44)	0.74
5 (R_f=0.54)	1.4		
Gaps[c]	3.0	Gaps	0.3

[a]Tryptophan solutions in 0.1 M sodium phosphate buffer were spiked with radioactive tryptophan of known activity and allowed to react with aqueous chlorine or chlorine dioxide solutions at a 7:1 disinfectant:tryptophan ratio for 24 hours. Any residual chlorine or chlorine dioxide remaining after the reaction was removed with sodium sulfite. The solutions were filtered and concentrated by resin adsorption using Amberlite XAD-2/8. The adsorbed compounds were eluted with acetone and concentrated. The acetone eluates were then fractionated on silica gel TLC plates. The different fractions detected by exposure to UV light were then scraped off and the radioactivity in the samples was counted by liquid scintillation counting.

[b]Percent radioactivity was determined by dividing the radioactivity count of each band by the total radioactivity applied to the TLC plates.

[c]The silica gel between the fluorescent compounds were also scraped off and radioactivity determined.

to the original material were obtained in all the fractions. The intensity of the color decreased from the origin to the least polar product. The dry samples were dissolved in and diluted with DMSO and assayed for mutagenic activity.

Aqueous chlorine. Mutagenic activity was detected in all the 5 bands of the TLC extracts in strain TA98 without metabolic activation (Table 21). From the relative mutagenicity values, band #4 appeared to be the most potent in this bacteria strain and closely followed by band #5. Toxicity was observed at 5 mg/plate for bands #2 and #3. When the S-9 mix was added, there was a sharp decrease in mutagenic activity in all the samples. Addition of the S-9 mix reduced the mutagenicities of the extracts and bands 2-4 were only weakly mutagenic while band #1 was not. Also bands 2 and 3 which showed some toxicity were no longer toxic (Table 21).

Toward strain TA100, band #1 was barely mutagenic at the highest dose tested while the rest of the samples exhibited potent mutagenic activity in the absence of the S-9 mix (Table 22). Bands #2 and 5, both of which showed blue fluorescence, were the most potent in showing mutagenic activity and were more so than the non-fractionated extracts. Bands #3 and 4 were also very potent but to a limited degree (Table 22). Thus the fractionation process was able to remove some of the toxic or antimutagenic components in the reaction mixture. In the presence of the S-9 mix, there was a major reduction in mutagenic activity such that band #1 tested negative and

Table 21. Mutagenicity of the TLC subfractions obtained from the Amberlite XAD eluates of the reaction products of aqueous chlorination of tryptophan using Salmonella typhimurium strain TA98[a]

Sample	Dose/plate	Number of revertants/plate[b] TA98 -S9	MR[c]	+S9	MR
Bacteria		27± 2		28± 7	
Bacteria + DMSO	25 μL	22± 2		26± 7	
Band #1 R_f=0					
	250 μg	22± 4	1.0*	31± 7	1.2*
	1 mg	33± 5	1.5	28± 2	1.1*
	5 mg	78± 6	3.5	30± 6	1.2*
Band #2 R_f=0.08					
	250 μg	44± 6	2.0	30± 7	1.2*
	1 mg	174±15	7.9	36± 5	1.4*
	5 mg	T[d]		56±10	2.2
Band #3 R_f=0.22					
	250 μg	26± 4	1.2*	28± 6	1.1*
	1 mg	118±10	5.4	34± 5	1.3*
	5 mg	T		71±19	2.7
Band #4 R_f=0.38					
	500 μg	62±10	2.8	26± 6	1.0*
	1 mg	138± 8	6.3	28±10	1.1*
	5 mg	598±59	27.2	62± 8	2.4
Band #5 R_f=0.54					
	250 μg	53± 6	2.4	30± 4	1.2*
	1 mg	111± 8	5.0	44± 5	1.7
	5 mg	418±20	19.0	89± 7	3.4

Table 21-continued.

[a] Aqueous chlorine was allowed to react with tryptophan (7:1 molar ratio) at room temperature for 24 hours. Any excess chlorine remaining after the reaction was removed with sodium sulfite and the reaction products were concentrated by Amberlite XAD adsorption using acetone as eluant. The XAD eluates were further fractionated by thin layer chromatography on silica gel G plates developed with a hexane:ethyl ether:acetic acid (50:50:1, v/v/v) solvent system. The fractions were then assayed for mutagenic activity in Salmonella typhimurium strain TA98.

[b]Results of duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the solvent controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

Table 22. Mutagenicity of the TLC subfractions obtained from the Amberlite XAD eluates of the reaction products of aqueous chlorination of tryptophan using Salmonella typhimurium strain TA100[a]

		Number of revertants/plate[b]			
		TA100			
Sample	Dose/plate	-S9	MR[c]	+S9	MR
Bacteria		120± 13		89± 14	
Bacteria + DMSO	25 μL	116± 6		95± 2	
Band #1 R_f=0					
	250 μg	113± 4	1.0*	111± 9	1.2
	1 mg	158± 15	1.4	110± 14	1.2*
	5 mg	321± 30	2.8	124± 20	1.3
Band #2 R_f=0.08					
	250 μg	225± 15	1.9	113± 18	1.2*
	1 mg	594± 72	5.1	144± 16	1.7
	5 mg	3987±186	34.4	430± 39	4.5
Band #3 R_f=0.22					
	250 μg	180± 19	1.6	126± 9	1.3
	1 mg	915± 22	7.9	166± 6	1.7
	5 mg	T[d]		522± 33	5.5
Band #4 R_f=0.38					
	500 μg	267± 26	2.3	130± 15	1.4
	1 mg	1420± 88	12.2	154± 11	1.6
	5 mg	T		648±113	6.8
Band #5 R_f=0.54					
	250 μg	177± 20	1.5	146± 15	1.2
	1 mg	1017± 96	8.8	213± 20	2.2
	5 mg	4128±419	35.6	728± 47	7.6

Table 22-continued.

[a]Aqueous chlorine was allowed to react with tryptophan (7:1 molar ratio) at room temperature for 24 hours. Any excess chlorine remaining after the reaction was removed with sodium sulfite and the reaction products were concentrated by Amberlite XAD adsorption using acetone as eluant. The XAD eluates were further fractionated by thin layer chromatography on silica gel G plates developed with a hexane:ethyl ether:acetic acid (50:50:1, v/v/v) solvent system. The fractions were then assayed for mutagenic activity in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided by that of the solvent controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Toxic.

bands 3 and 4 were mutagenic only at the maximum dose of 5 mg/plate. Also the toxicity in bands 3 and 4 was eliminated. The most potent mutagenic band (#5) was positive at two doses but with about a 5-fold decrease in mutagenic activity at the highest dose tested (Table 22).

For the purposes of the mutagenicity assessment of the products in band 5 re-developed in the second dimension, the TLC plates were divided into several portions. All portions (including both fluorescent and nonfluorescent bands) were scraped off separately, dissolved in acetone and dried. The dry samples were then taken up in DMSO and assayed for mutagenic activity. This assay was performed only in bacteria strain TA100 without metabolic activation. The results are presented in Table 23. High mutagenic activity was detected in only 2 fractions. Band #5b which did not show any fluorescence was mutagenic at the highest dose tested (Table 23). The only other band which showed higher mutagenic activity was band #5c (R_f=0.31) which showed blue fluorescence similar to the original material. This fraction showed a dose-related increase in mutagenic activity with very high mutagenicity ratios at the higher doses tested (Table 23).

Chlorine dioxide. Mutagenic activity was detected in all the TLC bands of chlorine dioxide reaction mixture in strain TA98. In all cases mutagenic activity was weak and not comparable to that of the aqueous chlorine samples (Table 24). As opposed to aqueous chlorine samples, no toxicities were

Table 23. Mutagenicity of the TLC fraction of the reaction products of the aqueous chlorination of tryptophan re-developed on a second plate[a]

Sample	Dose/plate	Number of revertants/plate[b] TA100 -S9	MR[c]
Bacteria		109± 11	
Bacteria + DMSO	25 μL	118± 8	
Band #			
5a (R_f=0)[d]	1.0 mg	103± 17	0.9*
	2.5 mg	141± 13	1.2
	5.0 mg	231± 18	2.0
5b (R_f=0.16)	1.0 mg	170± 35	1.4
	2.5 mg	216± 16	1.8
	5.0 mg	641± 31	5.4
5c (R_f=0.31)	1.0 mg	150± 7	1.3
	2.5 mg	627± 80	5.3
	5.0 mg	1520±103	12.9
5d (R_f=0.46)	1.0 mg	86± 6	0.7*
	2.5 mg	117± 7	1.0*
5e (R_f=0.62)	1.0 mg	93± 11	0.8*
	2.5 mg	102± 2	0.9*
	4.0 mg	105± 3	0.9*
5f (R_f=0.65)	1.0 mg	102± 6	0.9*
	2.5 mg	115± 2	1.0*
	5.0 mg	158± 15	1.3*
5g (R_f=0.75)	1.0 mg	86± 9	0.7*
	2.5 mg	84± 5	0.7*
	5.0 mg	104± 8	0.9*
5h (R_f=0.88)	1.0 mg	91± 9	0.8*
	2.5 mg	95± 5	0.8*
	5.0 mg	92± 8	0.8*

Table 23-continued

[a]Aqueous chlorine was allowed to react with tryptophan (7:1 molar ratio) at room temperature for 24 hours. Any excess chlorine remaining after the reaction was removed with sodium sulfite and the reaction products concentrated by Amberlite XAD adsorption using acetone as eluant. The XAD eluates were fractionated by thin layer chromatography on silica gel G plates developed with hexane:ethyl ether:acetic acid (50:50:1, v/v/v). A product from the above fraction was further separated on a second TLC plate using an eluant comprising hexane:methylene chloride:acetic acid (50:50:2, v/v/v). The fractions were then assayed for mutagenic activity in Salmonella typhimurium strain TA100.

[b]Results of duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided that of the controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Bands 5a, 5b, 5d and 5g did not show fluorescence therefore their R_f values were taken from the center of the band.

Table 24. Mutagenicity of the TLC subfractions obtained from the Amberlite XAD eluates of the reaction products of chlorine dioxide and tryptophan using Salmonella typhimurium strain TA98[a]

Sample	Dose/plate	Number of revertants/plate[b] -S9	MR[c]	+S9	MR
Bacteria		30± 7		29± 5	
Bacteria + DMSO	25 μL	28± 9		26± 7	
Band #A R_f=0					
	1 mg	47± 6	1.7	26± 2	1.0*
	2 mg	51± 5	1.8	33± 5	1.3*
	5 mg	79±14	2.8	29± 1	1.1*
Band #B R_f=0.13					
	1 mg	41± 2	1.5	27± 9	1.0*
	2 mg	56± 2	2.0	33± 5	1.3*
	5 mg	84± 3	3.2	33± 2	1.3*
Band #C R_f=0.31					
	1 mg	ND[d]		ND	
	2 mg	83± 4	3.0	33± 4	1.3*
	5 mg	132±15	4.7	60± 6	2.3
Band #D R_f=0.44					
	1 mg	40± 5	1.4*	27± 4	1.0*
	2 mg	49± 5	1.8	32± 4	1.2*
	5 mg	74± 7	2.6	28± 1	1.1*

[a]Aqueous chlorine dioxide was allowed to react with tryptophan (7:1 molar ratio) at room temperature for 24 hours. Any excess chlorine dioxide remaining after the reaction was removed with sodium sulfite and reaction products were concentrated by Amberlite XAD adsorption using acetone as eluant. The XAD concentrates were further fractionated by thin layer chromatography using silica gel G plates developed with a hexane:ethyl ether:methanol:acetic acid (63:30:7:2, v/v/v/v) solvent system. The fractions were then assayed for mutagenic activity in Salmonella typhimurium strain TA98.

[b]Duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided that of the solvent controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Not determined.

observed in these samples even at the highest dose of 5 mg/plate. Perhaps the strongest mutagen in these extracts was a narrow blue fluorescent band designated C. In the presence of the S-9 mix, mutagenic activity was abolished in all the extracts except band C.

In bacterial strain TA100, band B was weakly mutagenic at 5 mg/plate and C was mutagenic at 2 doses. Bands A and D were basically non-mutagenic (Table 25). No observable changes were observed on addition of the S-9 mix. Again bands B and C tested positive in the presence of the S-9 mix even though the activities were weak (Table 25).

Induction of Sister Chromatid Exchange by the TLC Fractions of the Reaction Products of Aqueous Chlorine or Chlorine Dioxide with Tryptophan

The mutagenic TLC subfractions of the reaction products of aqueous chlorine or chlorine dioxide with tryptophan were further evaluated for genotoxicity using the sister chromatid exchange assay. For the aqueous chlorine reaction products, bands 2 through 5 were used while in the case of chlorine dioxide only bands B and C were used. A high dose of 1 mg/mL, a low dose of 1 μg/mL and other intermediate doses were used for all the treatments. The results indicated that the reaction products in these bands were capable of significantly ($P=0.05$) increasing the sister chromatid exchange frequencies over the DMSO control (Table 26). Except for sample D, the

Table 25. Mutagenicity of the TLC subfractions obtained from the Amberlite XAD eluates of the reaction products of chlorine dioxide and tryptophan using Salmonella typhimurium strain TA100[a]

Sample	Dose/plate	Number of revertants/plate[b] -S9	MR[c]	+S9	MR
Bacteria		106±12		109± 8	
Bacteria + DMSO	25 μL	98± 7		94± 7	
Band #A R_f=0					
	1 mg	129±18	1.3	122± 9	1.3
	2 mg	118±22	1.2*	118±10	1.3
	5 mg	149±18	1.5	129± 9	1.4
Band #B R_f=0.13					
	1 mg	128±25	1.3*	122± 9	1.3
	2 mg	169±23	1.7	158±16	1.7
	5 mg	275±23	2.8	214±34	2.3
Band #C R_f=0.31					
	1 mg	ND[d]		ND	
	2 mg	281±35	2.9	250±13	2.7
	5 mg	532±65	5.4	489±12	5.2
Band #D R_f=0.44					
	1 mg	103±15	1.1*	109±10	1.2
	2 mg	123±14	1.3	106±10	1.1*
	5 mg	147±15	1.5	128±17	1.4

[a]Aqueous chlorine dioxide was allowed to react with tryptophan (7:1 molar ratio) at room temperature for 24 hours. Any excess chlorine dioxide remaining after the reaction was removed with sodium sulfite and reaction products were concentrated by Amberlite XAD adsorption using acetone as eluant. The XAD concentrates were further fractionated by thin layer chromatography using silica gel G plates developed with a hexane:ethyl ether:methanol:acetic acid (63:30:7:2, v/v/v/v) solvent system. The extracts were then assayed for mutagenic activity in Salmonella typhimurium strain TA100.

[b]Duplicate runs of 4 plates/dose. Means and standard deviations.

[c]Mutagenicity ratio is the number of revertants per test dose divided that of the solvent controls. Asterisk denotes not significantly different from control samples (P=0.05).

[d]Not determined.

Table 26. Sister chromatid exchange frequencies induced by the TLC subfractions of the reaction products of aqueous chlorine and chlorine dioxide with tryptophan[a]

Sample	Dose	SCE per cell[b]
DMSO	25 μL	6.90±2.1
MMS	0.002 μL	29.15±5.4
Aqueous chlorine		
Band #2, R_f=0.08	200 μg	11.75±2.6
	100 μg	11.16±2.9
	10 μg	7.75±1.9[c]
Band #3, R_f=0.22	200 μg	11.32±2.7
	100 μg	10.15±2.8
	10 μg	6.94±2.0[c]
Band #4, R_f=0.38	200 μg	8.70±2.6
	100 μg	8.32±2.4[c]
	10 μg	6.96±2.2[c]
Band #5, R_f=0.54	200 μg	11.74±2.5
	100 μg	9.50±1.7
	10 μg	7.15±2.2[c]
Chlorine dioxide		
Band #B, R_f=0.13	1 mg	10.60±2.5
	100 μg	8.10±2.5[c]
	10 μg	6.89±2.1[c]
Band #C, R_f=0.31	200 μg	10.94±2.5
	100 μg	9.85±2.1
	10 μg	6.84±1.7[c]

[a]Aqueous chlorine or chlorine dioxide were allowed to react with tryptophan (7:1 molar ratio) for 24 hours. The reaction mixture was concentrated by resin adsorption and the adsorbed compounds were eluted with acetone. The acetone eluates were further fractionated by thin layer chromatography on silica gel G plates. Compounds in the TLC fractions were extracted with acetone. Following proper processing, assayed for genotoxicity using the sister chromatid exchange assay in CHO cells.

[b]The data were tranformed logarithmically and analyzed for statistical significance using the Dunnett's test.

[c]Not significantly different from DMSO control sample.

highest doses tested (1 mg/mL) did not provide enough metaphases for chromosomal analysis. Bands 2 and 5 were the most genotoxic of the aqueous chlorine reaction products at the highest positive doses. The other aqueous chlorine and chlorine dioxide fractions also showed genotoxic activity. This is in line with the Ames mutagenicity data. Statistical analysis of the results using the Dunnett's test (Steel and Torrie, 1980) showed that all the samples were significantly different (P=0.05) from the control samples at the highest non-toxic doses tested. A test dose of 10 μg/mL was found to be non significantly different in all the treatments. Band #4 and #C were also not significantly different (P=0.05) from control samples (Table 26).

Identification of the Reaction Products

For the aqueous chlorine reaction products, the neutral methylene chloride and hexane extracts from the preliminary liquid-liquid extraction study as well as the highly mutagenic TLC subfraction (band #5) were analyzed by GC/MS. The methylene chloride and hexane extracts contained several compounds; some of which have previously been identified by other researchers. A reconstructed ion chromatogram of the products from the methylene extracts is presented in Fig. 8. Three indole derivatives, oxindole, isatin and indolyl-acetonitrile, and two quinoline-type compounds, norhaman and 3-hydroxyquinaldonitrile, were tentatively identified from

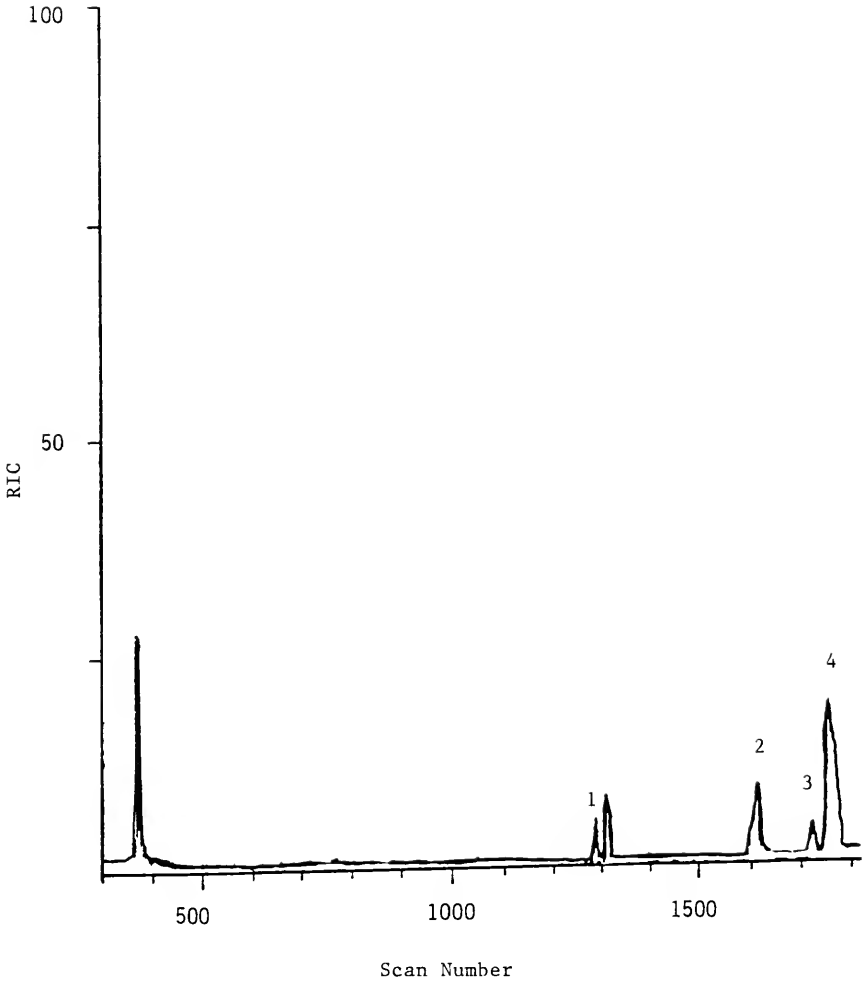

Figure 8. Reconstructed ion chromatogram of the methylene chloride extract of the reaction products of aqueous chlorine with tryptophan. 1) oxindole 2) indolylacetonitrile 3) isatin, and 4) norhaman.

this extract. The precipitate was analyzed by direct insertion probe and heated on the column but was found to be highly nonvolatile with the bulk of the materials coming up late in the program as combustion products. The only chlorinated compounds detected in this fraction resembled those of trisubstituted chlorophenols which probably arose from the water. Chlorophenols are used as wood preservatives and it is not uncommon to find them in water.

The mass chromatogram of the products from the TLC subfraction (band #5) is presented in Fig. 9. Three chlorinated compounds were present in this fraction. Two chloroketones, 1,1,3-trichloropropanone and 1,1,3,3-tetrachloropropanone and one quinoline compound, dichloroquinoline were tentatively identified in this fraction. Another TLC subfraction (band #4) was analyzed (Appendix) and found to contain four chlorinated compounds. Some of the compounds such as chloral have been identified in chlorinated humic acid solutions (Horth et al., 1987). Another compound similar to dichloroquinoline but with three chlorines attached was present in this fraction (Appendix).

The mass chromatogram of the weakly mutagenic TLC subfraction of the chlorine dioxide reaction products was a simple one containing only two compounds (Fig. 10). Computer analysis of the spectra of the compounds from this fraction with compounds in its library did find any close matches. The

spectra for these compounds resembled those 2,4-dimethyl pentanol and 2,4-dimethyl pentane.

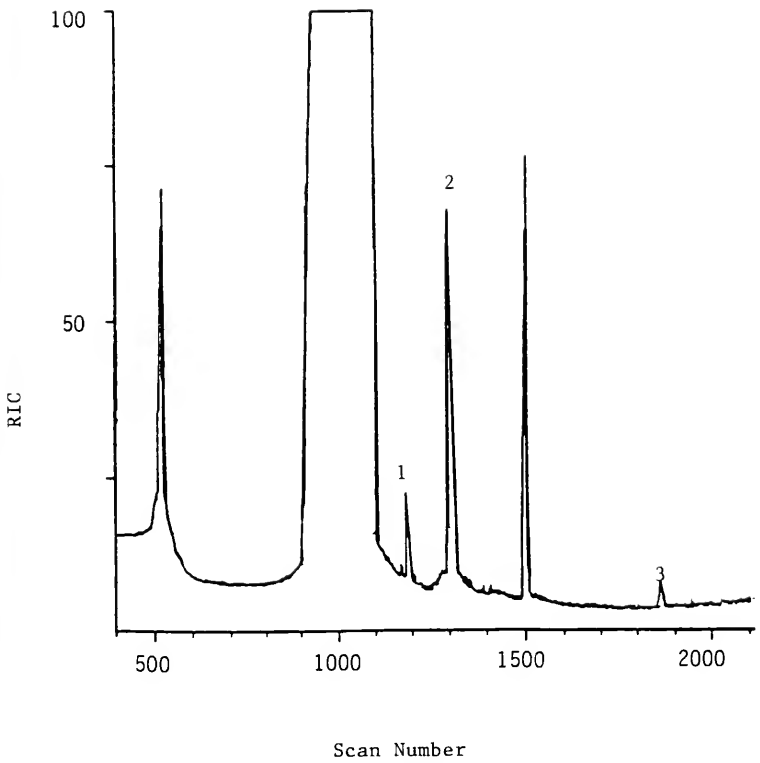

Figure 9. Reconstructed ion chromatogram of the of the thin layer chromatography subfraction of the reaction products of chlorine with tryptophan (band 5). 1) 1,1,3-trichloropropanone 2) 1,1,3,3-tetrachloropropanone, and 3) dichloroquinoline.

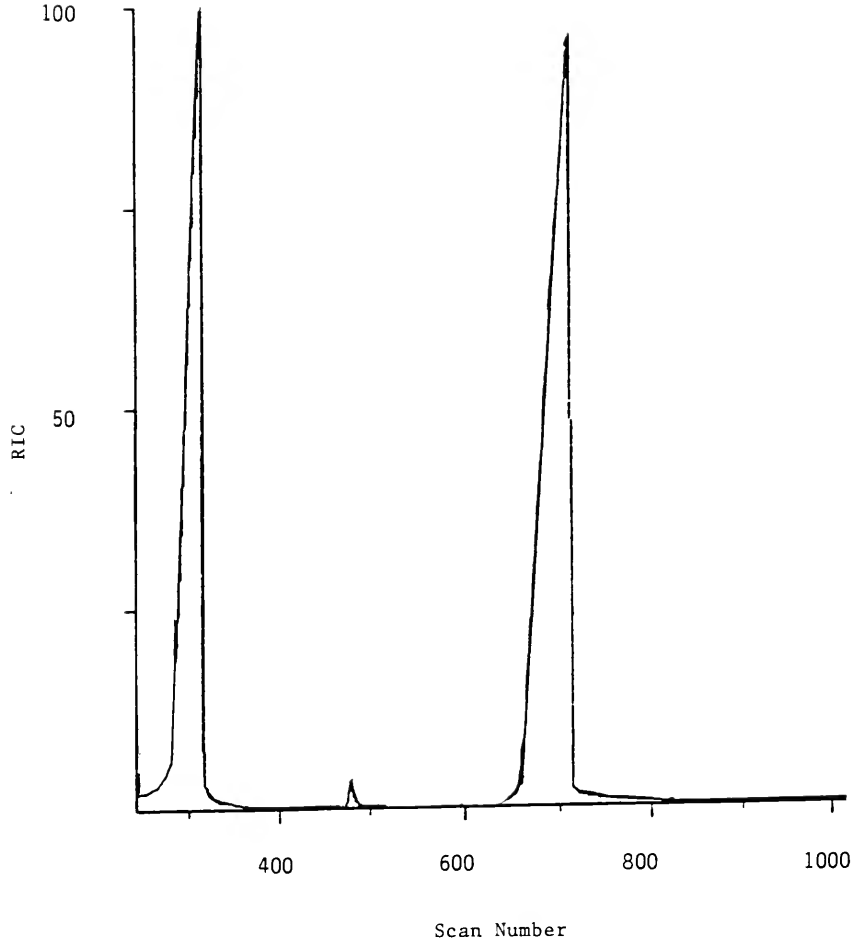

Figure 10. Reconstructed ion chromatogram of the thin layer chromatography subfraction of the reaction products of chlorine dioxide with tryptophan (band C).

CHAPTER V
DISCUSSION

Due to its efficiency, low cost, ease of application and adequate persistence in water, chlorine has been widely used since World War II for water disinfection (White, 1972). Because of its continued use, several studies have been conducted by different researchers in order to understand the chemistry and fate of chlorine in drinking water. As a result of these studies, researchers have gained a lot of information on the use and possible health risks posed by chlorine in drinking water treatment. Also the reactions of chlorine with amino acids in dilute aqueous solutions have been studied in some detail. The early studies on the chlorination of amino acids focused mainly on the identification of the reaction products (Dakin et al., 1916; Langheld, 1909), while more recent studies have concentrated on the genotoxic properties as well as identification of the mutagenic reaction products likely to be produced from such reactions (Horth et al., 1987; Trehy et al., 1986).

Reactions of Aqueous Chlorine and Chlorine Dioxide with Tryptophan

Tryptophan has been used as a model in different studies to investigate chloroform formation or mutagenic activity in aqueous chlorine solutions (Sussmuth, 1982; Morris and Baum, 1978). Kirk and Mitchell (1980) investigated the reactions of aqueous chlorine and tryptophan using different concentrations and observed the formation of a dark-colored precipitate upon reaction. Burleson et al. (1980) also observed a reddish color formation in their ether extracts.

In the present study, a dark-colored reaction mixture and precipitate were formed upon mixing tryptophan and aqueous chlorine solutions at three different molar ratios (chlorine: tryptophan, 1:1, 3:1 and 7:1). The precipitate continued to be formed even when the reaction products were concentrated by Amberlite XAD-2/8 adsorption and kept refrigerated. When the molar concentration of chlorine was increased more than 15-fold over that of the amino acid, color formation was not observed. Other researchers have studied similar reactions in model systems but did not report color formation (Horth et al., 1987; Trehy et al., 1986; Sussmuth, 1982). This is probably due to the fact that the amount of chlorine used in these studies was in excess of the amino acid which precluded color formation. Sussmuth (1982) bubbled 80 mL of chlorine gas into a solution containing 250 mg of tryptophan; Trehy and coworkers (1986) used a chlorine to amino acid ratio of 14.

At these ratios, color and precipitate formation were either not observed or not reported.

In the case of chlorine dioxide, very little information is available in the literature regarding its reactions with amino acids. Masschelein (1979) reported that tryptophan was very reactive with chlorine dioxide leading to the formation of reddish coloration. In this study, reddish coloration was observed when excess chlorine dioxide was reacted with tryptophan. The reddish color changed to a dark color when the reaction progressed. The amount of precipitate and intensity of color formed from the chlorine dioxide reaction mixtures were much less than that of aqueous chlorine. The only other report in the literature on the reaction of chlorine dioxide with tryptophan was published in 1957 by Fujii and Ukita who reported formation of complex reaction mixtures containing indigo red as well as unidentified polymerization products. Structurally similar indole compounds have been shown to react with chlorine dioxide under water treatment conditions (Lin and Carlson, 1984). Chlorine dioxide oxidation of indole produces oxindole and isatin as major products, while with 3-methylindole, 3-methyloxindole and o-formamidoacetophenone were produced as the major products. Color formation in the reactions between chlorine dioxide and tyrosine has been well documented and is used as a basis for the determination of chlorine dioxide concentrations in water (Hogden and Ingols, 1954).

In general methyl ketones or compounds oxidizable to these structures undergo base-catalyzed halogenation reactions leading to the formation of chloroform and other THM (Rook, 1974). The reactivity of tryptophan and structurally similar pyrrole compounds is due to the fact they are capable of active carbanion formation. Due to their reactive nature, such compounds are capable of rapid reaction with chlorine leading to formation of chloroform and other THM in a mechanism similar to the haloform reaction (Morris and Baum, 1978). According to Morris and Baum (1978), acetone or other simple methyl ketones react too slowly to account for chloroform formation under water chlorination conditions. For acetone, the computed half-life for haloform formation at neutral pH and at ambient temperature is almost one year. Compounds such as beta diketones and the pyrroles are capable of giving chloroform yields approaching one mole per mole of compound within a few hours (Morris and Baum, 1978). In model chlorination studies, Morris and Baum (1978) observed linear increases in chloroform formation when excess chlorine was reacted with tryptophan. The molar yields of chloroform in these studies were about 18% at pH 7.5 compared to about 100% at pH 11.0. Tryptophan reacted with chlorine under neutral or slightly acidic pH conditions with chloroform being liberated at elevated pH. Thus, tryptophan and structurally similar pyrrole compounds may account for some of the haloform formation under water chlorination conditions.

Mutagenicity Evaluation

Short-term *in vitro* bioassays have become very popular over the past decade or so primarily because they can be performed relatively easily and inexpensively compared to long-term carcinogenicity assays which often involve the use of several rodents and other mammals. Also, protests from animal groups have led to serious considerations about using alternate *in vitro* assays which do not require the use of test animals. The best known and perhaps most widely used short-term assay for detecting mutagenic and potentially carcinogenic compounds in the environment and industry is the Ames *Salmonella*/mammalian microsome assay. This is because it is relatively easy to perform and has a sizeable literature base (Tennant et al., 1987). The Ames test uses different strains of *S. typhimurium* bacteria which have been genetically modified to detect mutagens and potential carcinogens. The most sensitive and widely used bacteria, strains TA98 and TA100 are used to detect frameshift and base-pair substitution mutagens, respectively. The tests can also be performed with the addition of mammalian liver homogenate (rat liver S-9 fraction) which contains all essential drug metabolizing enzymes to simulate mammalian metabolism. Other recognized *in vitro* short-term assays include chromosomal aberrations, sister chromatid exchange and mouse lymphoma cells. Even though these assays have their inherent limitations (e.g.

false positives and negatives), they are nonetheless used extensively for screening chemical carcinogens and mutagens.

The original report of the Ames test which indicated a 90% correlation between carcinogenicity and mutagenicity (McCann et al., 1975), was later revised to about 83% by the same authors (Ames and McCann, 1981). More recently, the correlation has been shown to be much less than originally predicted. Tennant et al. (1987) have reported that test results from four in vitro short-term assays including the Ames test did not show concordance with rodent carcinogenicity data and that concordance of each assay was about 60%. There was, however, good agreement between the short-term tests in that when a chemical tested positive in one assay, it tended to be positive in the other three assays. As pointed out by the authors, short-term tests have been used successfully in certain areas where whole animal assays have not been used. For example short-term tests have been used for the analysis of complex mixtures of cooked meat, chlorinated water, air pollutants and for the early detection and identification of genotoxicants before manufacture. In summary, even though short-term tests are useful in their own way, they cannot replace whole animal assays completely.

Concentration of the reaction products

As stated earlier, the versatility of the Ames and other short-term tests lies in their ability to detect chemical carcinogens and/or mutagens in complex environmental mixtures.

Except for a few compounds e.g. chloroform, chemical mutagens are usually formed at the microgram per liter (ppb), or lower levels in chlorinated drinking water (Tardiff et al., 1978). It is therefore necessary to concentrate drinking water samples by several orders of magnitude for chemical analysis and/or genotoxicity assessment. Some researchers have reported mutagenic activity in unconcentrated water samples but the reported activities are generally weak (Kool et al., 1981a).

Several methods are available for achieving this concentration step prior to analysis. These include liquid-liquid extraction, freeze drying, reverse osmosis and resin or activated carbon adsorption/desorption. Method selection for sample concentration has been found greatly to influence the results of different mutagenicity data, and as such, values reported from different sources may not be directly comparable (Vartiainen et al., 1987). Of the sorption methods, those that have been very well documented are the Amberlite resins XAD-2, XAD-4, XAD-7 and XAD-8 (Daignault et al., 1988). In this study, rotary evaporation, liquid-liquid extraction and Amberlite XAD polymeric resin adsorption were used to concentrate the reaction mixtures for genotoxicity assessment.

Rotary evaporation

This method is generally not employed in water chlorination studies because these studies require large

volumes of water (50 to 100 L) (Monarca et al., 1985; Grabow et al.; 1981). Also, the method is tedious and time consuming and most of the volatile materials may be lost if samples are concentrated this way. In the present study, mutagenic activity was detected in the complex reaction mixtures containing equimolar concentrations of aqueous chlorine and tryptophan concentrated by rotary evaporation. Mutagenic activity was decreased in all the samples in the presence of the rat liver enzyme system. The binding of the S-9 mix to the sample may have decreased the quantity of test chemicals available for bacterial mutagenesis, or the production of less toxic metabolites via the mixed function oxidase enzyme system may have led to a decline in the mutagenic activity. These results are in agreement with previously reported data (Sussmuth, 1982). Also at high doses (300 μL) the number of revertants was decreased indicating that the chemicals were causing some toxicity to the bacteria. There is a million-fold range of differences in carcinogenic and mutagenic potency among various chemicals tested in female mice and the Ames mutagenicity assay, respectively (McCann, 1983). Thus, some compounds require much higher concentrations to show the same carcinogenic and/or mutagenic effects as others.

The compounds were mainly direct-acting, nonvolatile mutagens. Nonvolatile materials which are also direct-acting have been shown to be produced as a result of water

chlorination and account for a substantial portion of the mutagenic activity observed in drinking water concentrates (Grabow et al., 1981; Cheh et al., 1980). Direct-acting and promutagens have been detected in unconcentrated water from the lower Mississippi river which include both volatile and nonvolatile materials (Pelon et al., 1977). Volatile materials such as chloroform and other THM are also produced as a result of chlorination and may contribute to the overall mutagenic activity in drinking water, but for the purposes of this study they are not expected to account for any of the observed mutagenic activity.

Liquid-liquid extraction

Liquid-liquid extraction by mechanical mixing, for example by magnetic stirring, has been used by several researchers to concentrate organic compounds from aqueous solutions for mutagenicity assessment and/or chemical analysis (Vartiainen et al., 1987; Trehy et al., 1986; Grabow, 1981). Other methods include continuous liquid-liquid extraction or Ultra-Turrax mixing (Vartiainen et al., 1987). The results from these studies differ slightly regarding solvent selection and extraction time. Traditionally, organic solvents like methylene chloride, ethyl ether, pentane, hexane and ethyl acetate or a combination of these or other solvents immiscible with water are used for these extractions.

Type of solvent and extraction time. In preliminary studies, liquid-liquid extractions were employed using hexane, ethyl ether and dichloromethane to extract the reaction products from equimolar concentrations of aqueous chlorine-tryptophan mixtures after sample pH adjustment. The results indicated that ethyl ether was more suitable than either dichloromethane or hexane (Tables 4 and 5). These results are in agreement with other published data (Vartiainen et al., 1987; Trehy et al., 1986). Even though both hexane and dichloromethane were capable of extracting some mutagens, the overall mutagenic activity in these extracts was lower than the ethyl ether extracts. Higher concentrations of the reaction products were found to be highly toxic to the bacteria, especially the neutral ethyl ether and dichloromethane extracts (Tables 4 and 5). Similar toxicities have been observed in drinking water concentrates (Grabow et al., 1980).

The use of some organic solvents has inherent disadvantages in that some of them, for example dichloromethane, have been shown to be carcinogenic and/or mutagenic. Dichloromethane has been found to be mutagenic in the Ames test when assayed in an open container inside a desiccator, but nonmutagenic when incorporated into the agar (Nestmann et al., 1980). Dichloromethane was found to be non-mutagenic when assayed in the HGPRT forward mutation assay in CHO cells. However, it caused a slight increase in SCE frequencies in V9 cells (Nestmann et al., 1981). It has also

been reported to be carcinogenic but not teratogenic in male and female rats (NTP, 1982). The dichloromethane used in the present assay is not expected to contribute to any of the observed mutagenic activity since the samples were poured directly onto the agar plates and control samples of the dichloromethane solvent alone were non-mutagenic.

Another concern about the use of some organic solvents in sample concentration by liquid-liquid extraction is the possibility of the formation of toxic metabolites from the reaction of residual chlorine with the solvent.

Vartiainen and coworkers (1987) used liquid-liquid extraction either with magnetic stirring or Ultra-Turrax mixing to concentrate Finnish drinking water. They reported that the concentrates obtained were mutagenic only in the presence of salt in the extraction procedure. Other researchers have used liquid-liquid extraction by Ultra-Turrax mixing without addition of salt and detected mutagenic activity in their extracts (Grabow et al., 1981). Vartiainen and colleagues (1987) also reported that continuous liquid-liquid extraction with ethyl ether and 20% salt was more efficient than using dichloromethane under similar conditions. In their studies, increased mutagenic activity was observed with longer extraction times (up to 48 hours). In the present study, no salt was added and only mechanical mixing with a magnetic stirrer was used. In the reaction mixtures with aqueous chlorine, higher mutagenic activity was observed at

the 1:1 ratio than at the 3:1 ratio. However, mutagenic activity was highest at the 7:1 ratio (Table 8). The decrease in activity at the 3:1 ratio may be due to the formation of less mutagenic products at this ratio. Similar results were observed for the three ratios of chlorine dioxide and tryptophan. However, the differences between the 1:1 and 3:1 ratios were not specific (Table 10). At high concentrations (5 mg/plate), toxicity was detected in the aqueous chlorine but not chlorine dioxide extracts. Bacteria strain TA98 was found to be more sensitive than TA100 indicating that the ethyl ether extracts were mainly frameshift mutagens even though some base-pair substitution mutations were also detected (Tables 8-11). Salt is usually added to liquid-liquid extracts to aid in the separation of phases and thereby increase recovery. It is not known whether addition of salt would have increased the efficiency of the solvent extractions and thereby the mutagenic activity.

Vartiainen et al. (1987) have reported that magnetic stirring for 15 minutes recovered only 5% of the mutagenic activity in Finnish water while stirring for 4 hours increased mutagen recovery to 25%. According to the authors, the most straightforward method of magnetic stirring did not give good results. However, this was not the case with our samples since the mutagenicity results from the liquid-liquid extractions correlated well with those of the XAD resin adsorption. Trehy et al. (1986) reported that 15 minutes

extraction time was adequate for the identification of components of interest from aqueous chlorine-tryptophan reaction mixtures. The authors also reported that ethyl ether was more suitable for solvent extraction of chlorination reaction products than pentane.

Effect of pH. pH is an important parameter to be considered in the separation of different classes of compounds by their acid/base behavior in drinking water prior to toxicological evaluation or chemical analysis. The order of the pH adjustment steps is essential since most of the mutagenic activity of the acid/neutral fraction is destroyed at high (alkaline) pH (Vartiainen et al., 1987). In this study, the neutral compounds were extracted before the acid or alkaline fractions which were also extracted separately (Fig. 5). The sequence of extraction which is similar to other published reports (Grabow et al., 1981), was expected to prevent the destruction of acidic compounds under alkaline conditions. Most of the mutagenic activity was detected in the neutral and acidic fractions (Tables 4 and 5). Some mutagenic activity was also detected in the alkaline fraction but was relatively weak. These findings are in agreement with other water chlorination results (Grabow et al., 1980).

In general, most of the observed mutagenic activity in drinking water samples have been found in the neutral fraction (Kool et al., 1982; Maruoka and Yamanaka, 1982). This is important from the standpoint of safety because water destined

for human consumption is at or around a pH of neutrality. Mutagenic activity is also associated with acidic compounds as well (Monarca et al., 1985; Holmbolm et al., 1984). Basic compounds usually account for very little or no mutagenic activity in drinking water. According to Maruoka and Yamanaka (1982), even though the level of extractable basic compounds in water is low (about 5%), these may probably contain some of the strongest mutagens.

Amberlite XAD resin adsorption

Amberlite XAD polymeric resins have received much attention in recent years because they have been used to concentrate minute quantities of organic compounds from large volumes of water. They have also been used for the concentration of mutagens and carcinogens from human urine (Yamasaki and Ames, 1977). Amberlite XAD resins are used widely because the method is rapid, easy to perform and requires much less organic solvent than liquid-liquid extractions. The resins are produced by Rohm and Haas (Philadelphia, PA) in different forms and polarities and with different physical properties. Amberlite XAD-2 is a non-polar polystyrene divinylbenzene copolymer, while XAD-8 is a methyl methacrylate polymer of intermediate polarity (Rohm and Haas, 1978 Technical Bulletin). Some of the physical properties of these resins are listed in Table 27.

Different researchers have studied mutagen formation in drinking water and model reactions using Amberlite XAD resin

Table 27. Some physical properties of Amberlite XAD-2 and XAD-8 resins[a]

Resin	Chemical nature	Surface area (m^2/g)	Polarity	Average pore diameter (Angstrom)	Mesh size
XAD-2	Polystyrene	330	Nonpolar	90	20-50
XAD-8	Acrylic ester	140	Intermediate	250	25-50

[a]Rohm and Haas Technical Bulletin, 1978.

concentration. Amberlite XAD-2 resins have been used alone (Horth et al., 1987; Tan et al., 1987b), or in combination with Amberlite XAD-8 (Meier et al., 1987; Ringhand et al., 1987). Amberlite XAD-4 resins have also been used alone (Vartiainen et al., 1987) or in combination with XAD-8 (Kool et al., 1981b). Other Amberlite resins such as XAD-7 have been used with varying success (Grabow et al., 1981). In the present study, the use of Amberlite XAD-2 and XAD-8 (1:1 mixture) in a single column was found to be more efficient (45-48% recovery) than using either resin alone (38% recovery) (Table 12).

The results of the Amberlite XAD resin adsorption study indicated that XAD-2/8 resins (1:1 mixture) were effective in concentrating mutagens in our model reaction mixtures of aqueous chlorine and chlorine dioxide with tryptophan. Both types of chlorination showed linear dose-related increases in mutagenic activity. With aqueous chlorine, the mutagenicity results confirm other studies in which Amberlite XAD-2 concentrating procedures were used (Horth et al., 1987; Tan et al., 1987b). However, this is the first report to show that increasing the ratio of aqueous chlorine over tryptophan, leads to an increase in mutagenic activity (Table 16). Under the conditions of this study, the highest mutagenic activity was detected in the 7:1 chlorine to tryptophan ratio (Table 16). In the case of chlorine dioxide, we have previously shown that its reaction with tryptophan (1:1 molar ratio)

leads to mutagen formation in the Ames test (Sen et al., 1989). The present study confirms our previous results that chlorine dioxide and tryptophan reaction mixtures (1:1 molar ratio) are mutagenic. Also, this study has shown that increasing the concentration of chlorine dioxide leads to increased mutagenic activity. These are the only studies showing that the reaction products of chlorine dioxide with tryptophan are mutagenic. Like other chlorination studies, the addition of the S-9 mix led to a decrease in the mutagenic activity. The only other report in the literature regarding mutagenicity studies of chlorine dioxide-tryptophan reaction products was by Tan et al. (1987b). Their results indicated that the reaction products of chlorine dioxide with tryptophan were not mutagenic at a 1:1 molar ratio. The discrepancies observed in this study and Tan's work may be due to the fact that Tan et al. used only Amberlite XAD-2 for concentrating their reaction products at pH 6.0 with ethyl ether elution. As a result, they recovered less than 12% of the radioactivity applied to their column. In this study, the use of both Amberlite XAD-2 and XAD-8 in one column with sample pH adjustment to pH 2.5 allowed for better recovery (about 60%) of the reaction products. Also, acetone, methanol and ethyl ether were used as eluants even though they were analyzed separately for mutagenic activity.

Type of eluant. Different eluants such as methanol, ethyl ether, acetone, ethyl acetate and DMSO are used to desorb

organic compounds from XAD resins. Of these, ethyl ether is perhaps the most widely used (Daignault et al., 1988). Due to its relatively nonpolar nature, ethyl ether allows for some polar interactions to occur via the oxygen and has found many applications for desorbing relatively nonpolar compounds from resin columns. It, however, has some disadvantages including flammability and production of side products such as 2-epoxypropane, hexane, hex-1-ene and 2-methyl pentane (Scott et al., 1984). Even though it may be suitable for desorbing some classes of compounds, ethyl ether is not suitable for others. Tan et al. (1987b) recovered less than 12% of their chlorination reaction products from XAD-2 columns, while Kool et al. (1981a) were able to recover only about 20% of the mutagenic activity from their XAD-4/8 resin column with ethyl ether. Subsequent elution with either DMSO or acetone yielded the remaining mutagenic activity (Kool et al., 1981a).

Artifact formation. Amberlite XAD-4 resins have been reported to produce more artifacts than XAD-2 or XAD-8 resins when used for concentrating chlorination reaction mixtures, especially when free chlorine is present in the samples (Sweeney and Cheh, 1985; Hunt and Pangaro, 1982). Generally, artifacts, which include naphthalenes, alkyl benzenes, phthalate esters, styrenes, and a host of other compounds have been associated with the resin manufacturing process (Daignault et al., 1988; James et al., 1981). Amberlite XAD-4 has been reported to produce more artifacts because of its physical

characteristics. With a high porosity and surface area (750 m^2/g), XAD-4 resins are believed to trap more artifacts than either XAD-2 (330 m^2/g) or XAD-8 (140 m^2/g) during manufacture (Daignault et al., 1988). Different theories have been put forward to explain artifact release from resin beads, all of which involve release of artifacts from the interstitial spaces deep within the beads (Daignault et al., 1988). Artifacts are thought to arise due to resin drying therefore resins are stored in a solvent, usually methanol (Junk et al., 1974). Sudden changes in solvent between aqueous-organic and back to aqueous have been found to correlate with artifact release. A study by Scott et al. (1984) showed that interaction of methanol and water with resin resulted in heat production with subsequent bead rupture and artifact release.

It has been reported that part of the mutagenicity in drinking water concentrated by Amberlite XAD adsorption may be actually due to artifacts from the resins or by reaction of chlorine with the resin (Daignault et al., 1988). In the present study, Amberlite XAD-2 and XAD-8 which have been reported to yield fewer artifacts, were used. Procedural blanks containing aqueous chlorine or chlorine dioxide alone in sodium phosphate buffer were run through the resins after removing any residual chlorine or chlorine dioxide with sodium sulfite. Also tryptophan blanks in phosphate buffer were passed through the XAD-2/8 resins to determine if artifacts from the resins contributed to mutagen formation. None of the

procedural blanks was found to be mutagenic (Table 15). These results and those of the liquid-liquid extractions confirm that mutagenic activity in our samples was not due to artifact formation. Studies by other workers have shown that distilled water blanks did not produce mutagens in XAD-2 or XAD-7 resins (Loper et al., 1983; Williams et al., 1982).

The effect of pH. pH is an important parameter which has been shown to affect the degree and efficiency of resin adsorption (Ringhand et al., 1987, Jolley, 1981). Because Amberlite XAD resins are nonionic, ionic organic compounds are adsorbed more efficiently on the beads at a pH at which ionization is suppressed. Neutral compounds on the other hand, are adsorbed irrespective of pH (Ringhand et al., 1987). Also, acidic and basic compounds can be adsorbed and desorbed very efficiently with simple pH manipulation (Daignault et al., 1988). In this study, pH adjustment of the reaction mixture to pH 2.5 prior to Amberlite XAD adsorption was found to increase product recovery when compared to adsorption at neutral pH (Table 13). This indicates that mutagenic activity in the reaction mixtures is mainly due to acidic/neutral compounds. These results confirm other water chlorination results in which more mutagens were observed under conditions of acidic than slightly basic or alkaline pH (Ringhand et al., 1987).

Effect of dechlorinating agents. Several agents such as sodium sulfite, sulfur dioxide, sodium thiosulfate and other sulfur compounds have been used to dechlorinate water samples

prior to sample concentration for mutagenicity evaluation. Dechlorinating agents are used to remove excess chlorine in order to prevent any reaction of chlorine with the concentrating agent such as organic solvents or Amberlite XAD resins. They are also used to stop the chlorination reaction. The use of these agents for chlorine removal has been reported to reduce the mutagenic activity of drinking water concentrates (Wilcox and Denny, 1985; Cheh et al., 1980). Cheh and colleagues reported that total removal of chlorine with sulfite caused a substantial reduction (50-80%) in the mutagenic activity of their XAD-4 resin concentrates of drinking water. Partial dechlorination (0.5 mg/L residual) has been reported to have very little or no effect on the mutagenic activity in drinking water concentrates (Wilcox and Denny, 1985); therefore, several researchers are using this approach to study mutagen formation in drinking water (Horth et al., 1987).

In water treatment plants, partial dechlorination is used to remove part of the applied chlorine in order to leave a residual for continued disinfection throughout the distribution system (Wilcox and Denny, 1985). Partial dechlorination in the final stage of water treatment also reduces the taste of chlorine in drinking water and makes it acceptable to the consumer. This practice will likely have no effect on the mutagenic activity in finished drinking water since the level of mutagens will still be high. In order to

circumvent the dechlorination problem, some researchers use longer reaction times to allow excess chlorine to evaporate, or bubble nitrogen gas through the mixture to facilitate excess chlorine removal (Ringhand et al., 1987; Vartiainen et al., 1987; Rapson et al., 1985).

In the present study, reaction of aqueous chlorine or chlorine dioxide with tryptophan at the 7:1 ratio for 24 hours usually left very little chlorine residual which was dechlorinated with sodium sulfite. Whether the sulfite was responsible for reducing some of the mutagenic activity in our samples is not known since mutagenic activity was highest in those dechlorinated samples, i.e., the 7:1 aqueous chlorine or chlorine dioxide:tryptophan reaction mixtures.

Fractionation of the reaction products

In drinking water chlorination studies, several difficulties arise in sample fractionation for mutagenicity assessment due to the diverse and complex nature of the chemicals found in drinking water. As a result, very few researchers have attempted further product fractionation for mutagenicity studies. In most cases, the amount of recovered mutagenicity does not equal the total mutagenic activity in the samples prior to the fractionation step. This is probably due to the fact that since organic mutagens are formed in very minute quantities, some small losses will severely impair the results. Several chromatographic techniques are available for

sample fractionation or separation of which HPLC is the most popular.

TLC separation. Good resolution of the reaction products (XAD eluates) was achieved by using TLC separation on silica gel plates. This mode of separation is equivalent to normal phase HPLC separation and the advantage of the TLC is that for mutagenicity evaluation, there is no dilution of the sample by the mobile phase. In this study, five fluorescent compounds or mixtures of compounds were obtained under UV light (366 nm) when excess aqueous chlorine was reacted with tryptophan (7:1 molar ratio). Development of band #5 of the aqueous chlorine extract on a second TLC plate revealed that the fluorescent bands were composed of a mixture of compounds (Table 23). Also, excess chlorine dioxide reacted with tryptophan to obtain four fluorescent bands which were also probably composed of a mixture. The fluorescent compounds were probably photosensitive because some loss of fluorescence was observed when the compounds were exposed to light. Also, leaving the plates in the dark for an extended period of time (about 48 hours) led to about 90% loss of fluorescence. This is the first report in which the reactions of aqueous chlorine and chlorine dioxide have been shown to produce fluorescent compounds with tryptophan. Other fluorescent compounds have been reported to be produced when tryptophan is treated with sodium nitrite under acidic conditions (Ohara et al., 1988).

Tryptophan has a UV absorbance at 280 nm which is used spectrophotometrically to determine protein concentrations in protein assays. Reactions of tryptophan with these chlorine compounds led to a strong UV absorbance in the long wavelength range (366 nm) and a weak absorbance in the short wavelength range (254 nm).

The TLC subfractions of the aqueous chlorine and tryptophan reaction products at the 7:1 ratio, gave the strongest mutagens. The least polar fraction band #5 which showed blue fluorescence was the most mutagenic. Other relatively polar compounds showing green fluorescence (bands #3 and #4) were also mutagenic (Tables 21 and 22). Further separation of the products from the highly mutagenic band #5 on another TLC plate produced one fraction which was highly mutagenic. The mutagenic activity in the latter which also showed blue fluorescence was about 33% of that of band #5 (Table 23). The difference between the TLC fractions and the original material, i.e. XAD and liquid-liquid extraction samples, is that the original samples were mainly frameshift mutagens (TA98) while the TLC fractions were mainly base-pair substitution mutagens (TA100) (Tables 16 and 21). Determination of product distribution in the TLC fractions using radioactivity indicated that most of the product (about 80%) was in the polar fraction (bands #1 and 2). The highly mutagenic fraction (band #5) accounted for less than 2% of the total radioactivity in the TLC fractions (Table 20). Further

fractionation of this band led the isolation of a potent mutagenic band which is expected to account for no more than 1% of the total mutagenicity in the reaction products. These results were expected since organic mutagens are usually formed at very low levels (μg/L or ppb) in drinking water.

In the case of the chlorine dioxide reaction products, weak mutagenic activity was detected in one polar (band B) and another slightly polar fraction (band C). Again, most of the radioactivity (93%) was in the polar fractions (bands A and B) (Table 20). Mutagenicity in these extracts was also mainly base-pair substitution mutagens which were relatively weak (Tables 24 and 25). The only fraction in these samples which can be considered mutagenic (band C) represented only about 2% of the applied material (Table 20). This mutagenic fraction was not deemed potent enough to warrant further fractionation.

Comparison of liquid-liquid extraction and Amberlite XAD-2/8 adsorption

This study was designed such that liquid-liquid extraction and Amberlite XAD resin adsorption could be compared directly. The reaction mixtures, usually 2 liters, were split into two portions with one-half for resin adsorption and the other half for liquid-liquid extraction (Fig. 6). The different doses used for each mutagenicity assay preclude any direct comparison. However, based on the raw data, it is evident that Amberlite XAD-2/8 resin

adsorption was more efficient than liquid-liquid extraction of the aqueous chlorine-tryptophan reaction mixtures (Fig. 11a). In this figure, the amount of sample used in the mutagenicity assay as processed for the liquid-liquid extraction was equivalent to 1 mg dry extract/plate, while the amount used as prepared by acetone elution of the XAD resins was equivalent to 100 μL/plate, or 0.1 mg/plate. On a weight-to-weight basis, the liquid-liquid extracts were about ten-fold more concentrated than the resin eluates. Statistical analysis of the data using the Student's t-test and comparing the individual ratios i.e. 1:1 resin versus 1:1 liquid-liquid extraction indicated that the resins concentrated significantly ($P<0.05$) more mutagens than the liquid-liquid extractions at all the levels tested (Fig. 11a). At the 1:1 aqueous chlorine to tryptophan ratio, liquid-liquid extraction produced about 49% the number of revertants in the XAD eluates, while at the 3:1 ratio, the number was less (about 28%). When the ratio of chlorine was increased further to 7:1, the total number of revertants in the liquid-liquid extracts increased to about 76% of that of the acetone eluates from the resins. Similar results were obtained for the chlorine dioxide-tryptophan reactions (Fig. 11b).

Two studies in the literature have compared liquid-liquid extraction and Amberlite XAD resin adsorption for concentrating mutagens in drinking water. Grabow et al. (1981) reported that liquid-liquid extraction by Ultra-Turrax

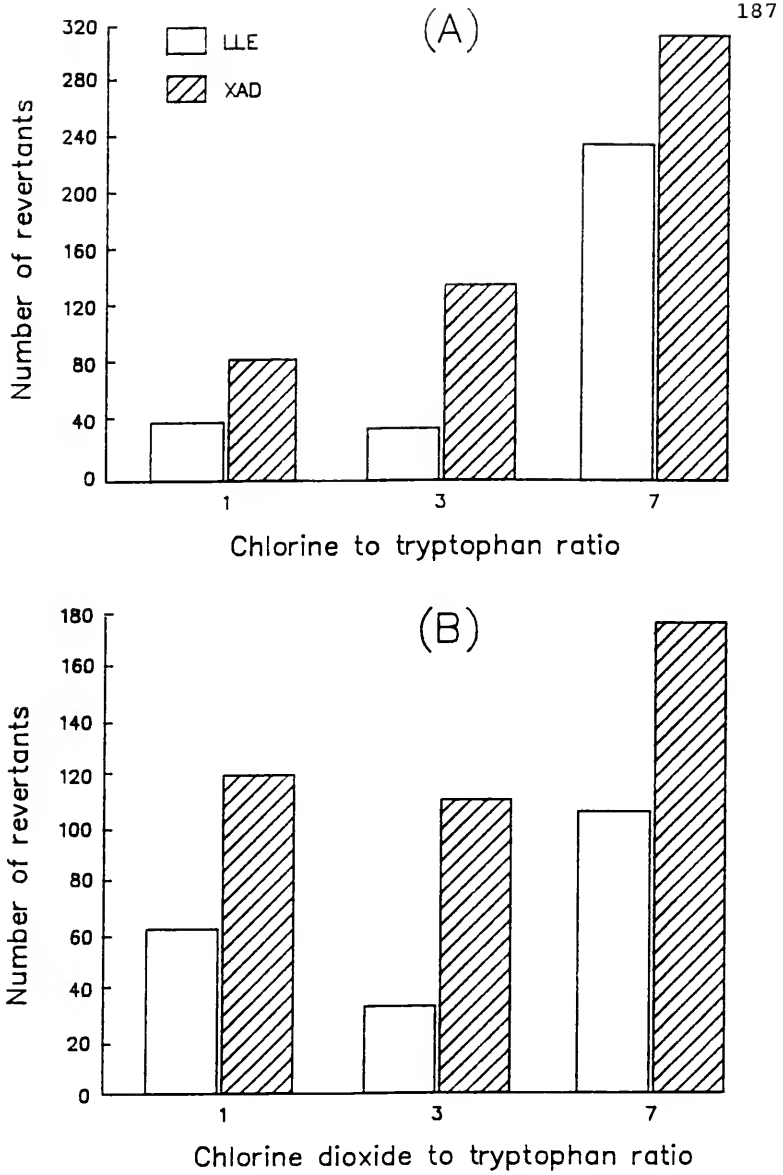

Figure 11. Comparison of liquid-liquid extraction (LLE, dose 1 mg/plate) and Amberlite XAD 2/8 adsorption (XAD, dose 100 μL/plate). A) aqueous chlorine and B) chlorine dioxide.

homogenization for 5 minutes using dichloromethane was superior to Amberlite XAD adsorption. Vartiainen and coworkers (1987) also compared liquid-liquid extraction by magnetic stirring, continuous liquid-liquid extraction for 24 or 48 hours, Ultra-Turrax mixing and Amberlite XAD resin adsorption. They reported that liquid-liquid extraction by magnetic stirring for 15 minutes and Ultra Turrax mixing were not efficient. Continuous liquid-liquid extraction was found to be comparable to XAD resin adsorption, especially when extraction times were longer (up to 48 hours). The results of the present study support the findings of Vartiainen et al. (1987) since liquid-liquid extraction by magnetic stirring for 24 hours gave results slightly lower than those obtained by resin adsorption (Fig. 11). The present results do not support those of Grabow and coworkers. Their extraction time was relatively short, and also, they used very large volumes of water (100 L) compared to organic solvent (520 mL). From these studies, it appears that at low chlorine or chlorine dioxide to tryptophan ratios (1:1 or 3:1), Amberlite XAD-2/8 adsorption is superior to liquid-liquid extractions. However, at high chlorine or chlorine dioxide to tryptophan ratios (7:1 molar ratio), liquid-liquid extractions tend to approach Amberlite XAD-8/2 adsorption (Fig. 11).

Comparison of aqueous chlorine and chlorine dioxide

A comparison of the mutagenic activities of the two disinfectants indicated that at the 1:1 molar ratio, chlorine

dioxide showed higher mutagenic activity than aqueous chlorine for both Amberlite XAD adsorption (Fig. 12a) and liquid-liquid extractions in both strains of bacteria (Fig. 12b). For comparative purposes, a dose of 100 μL/plate was used for the Amberlite XAD-2/8 eluates and 1 mg dry extract/plate for the liquid-liquid extracts. Statistical analysis of the data at this ratio showed that the chlorine dioxide reaction products were significantly ($P<0.05$) more mutagenic than the aqueous chlorine reaction products. At the 3:1 ratio, the order of mutagenic activity was somewhat reversed. The mutagenic activity was higher in most of the aqueous chlorine reaction mixtures than those of chlorine dioxide. Statistical analysis of the data indicated however that at this ratio, the aqueous chlorine reaction products were significantly more mutagenic than the chlorine dioxide reaction products in bacteria strain TA98 and the order was reversed in strain TA100 for the Amberlite XAD eluates (Fig. 12a). In the liquid-liquid extracts, the results were not statistically significant (Fig. 12b). Finally at the 7:1 ratio, the reaction products from aqueous chlorine showed significantly higher mutagenic activity than chlorine dioxide in both strains of bacteria for both concentration methods (Fig. 12).

At the low disinfectant to amino acid ratios, (i.e., 1:1 and 3:1), chlorine dioxide reaction products appeared to be more potent than the aqueous chlorine in most instances. However, when the concentration of disinfectant was increased

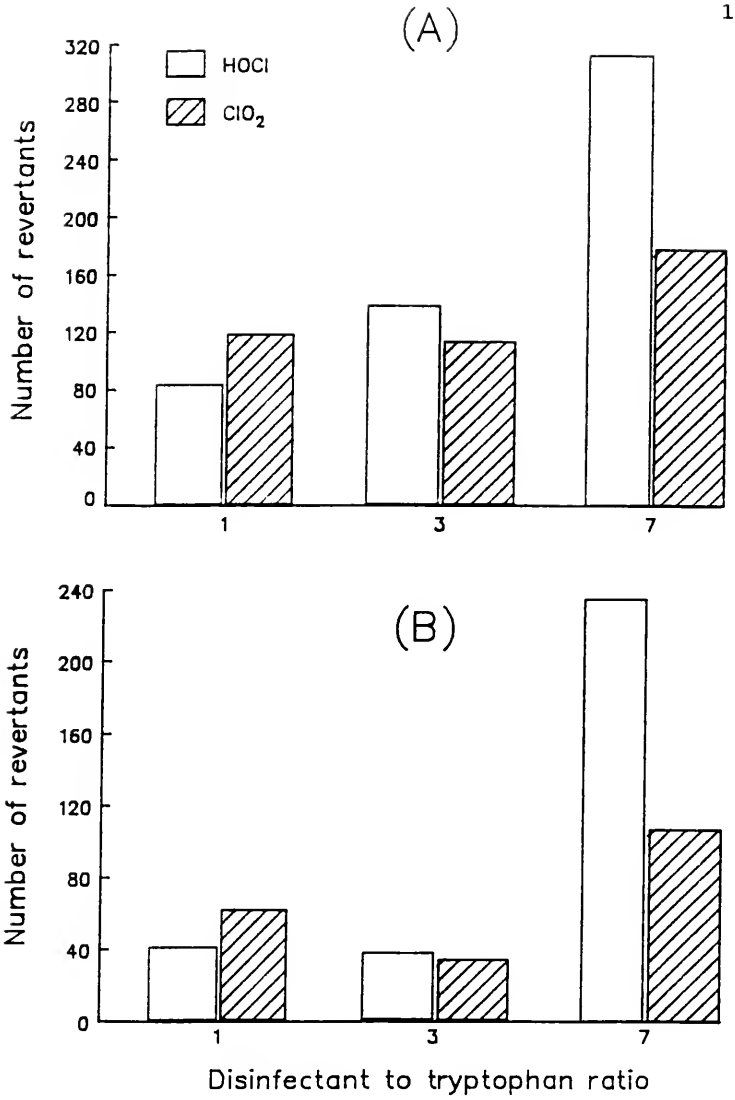

Figure 12. Comparison of aqueous chlorine and chlorine dioxide. A) Amberlite XAD 2/8 adsorption (XAD, dose 100 μL/plate), and B) liquid-liquid extraction (dose 1 mg/plate).

further (7:1 molar ratio), the aqueous chlorine reaction products became more potent than those of chlorine dioxide (Fig. 12). The reason for these differences may be explained by the fact that at the low disinfectant ratios, mainly oxidation reaction products are produced. The mutagenicities of the oxidation products have not been studied in any detail. However, at the highest disinfectant ratio, chlorine dioxide produces more oxidation byproducts while aqueous chlorine produces chlorinated reaction products which are more toxic and/or mutagenic.

Different researchers have compared the mutagenic activities of aqueous chlorine and chlorine dioxide reaction products in model systems and in drinking water. In most of the cases aqueous chlorine produces significantly more mutagens than chlorine dioxide (Kool et al., 1985), however, at high chlorine dioxide concentrations (5-15 mg/L), direct-acting mutagens have been shown to be produced (de Greef et al., 1980). Kool and colleagues (1985) and de Greef et al. (1980) showed dose-related increases in mutagenic activity at increasing chlorine dioxide concentrations in treated water, especially in S. typhimurium strain TA98.

Other reports in the literature are somewhat conflicting with regard to the mutagenic activity of chlorine dioxide reaction products. Rapson et al. (1985) investigated the mutagenic activity of the reaction products of aqueous chlorine or chlorine dioxide with tyrosine at increasing

disinfectant concentrations. Their results indicated that mutagenicity peaked at 4 equivalents of chlorine per mole of tyrosine. The results of the present study are in agreement with these findings. Also, according to Rapson and colleagues (1985) replacement of chlorine with chlorine dioxide produced no mutagens but this study showed otherwise.

From the results of the TLC subfractionation it is clear that aqueous chlorine reaction products were more potent mutagens than those of chlorine dioxide. None of the TLC subfractions of the aqueous chlorine and chlorine dioxide reaction products appeared similar therefore the mutagenic activities cannot be compared directly. From their relative mutagenicity values the least polar of the aqueous chlorine subfractions (band #5) had a mutagenicity ratio of 35.6 and 19.0 for bacterial strains TA100 and TA98, respectively, while the corresponding values for the chlorine dioxide subfractions were 1.5 and 2.6 at the highest doses tested. The most mutagenic of the TLC subfractions of the chlorine dioxide reaction products had mutagenicity ratio values of 4.7 and 5.4 for TA98 and TA100, respectively. Other strong mutagens with very high mutagenicity ratios were detected in the TLC subfractions of the aqueous chlorine samples while the overall mutagenicity ratios in the chlorine dioxide subfractions were relatively low (Tables 22 and 25).

Induction of Sister Chromatid Exchange by the TLC Subfractions of the Reaction Products of Aqueous Chlorine and Chlorine Dioxide with Tryptophan

Sister chromatid exchange (SCE) has received much attention in genetic toxicology because of its ability to detect S-dependent DNA-damaging agents (Latt et al., 1981). The methodology requires the substitution of thymidine by 5-bromodeoxyuridine (BrdU) or some other halogenated deoxynucleotide for at least one of two cycles of DNA synthesis. Chromosomes which replicate in the presence of BrdU for two cell cycles contain one unifiliary substituted and one bifiliary substituted chromatid. These differences can then be used to differentially stain the chromatids. The molecular basis of its induction has not been fully elucidated but it is thought to involve the breakage and joining of both DNA duplexes (one in each sister chromatid) present in a eukaryotic cell after DNA synthesis. Induction of SCE indicates a possible mutagenic and/or carcinogenic event. Like the Ames assay, SCE assays can be performed with or without the addition of the S-9 mix prepared from the livers of rats treated with Aroclor 1254. Also both assays are recommended for prescreening of chemicals for carcinogenic and/or mutagenic activity.

The results of the SCE assay in the present study indicated that most of the subfractions of the reaction of both aqueous chlorine and chlorine dioxide with tryptophan were capable of increasing the SCE frequencies over those of

the control samples (Table 26). There was a linear dose-related increase in SCE frequencies at all the nontoxic doses for both aqueous chlorine and chlorine dioxide reaction products. Except for band B of the chlorine dioxide subfractions, higher doses (1 mg/mL) of the other fractions were found to be cytotoxic and did not provide enough metaphases for chromosomal analysis. The S-9 mix was not included in the assay system because it was previously shown from the Ames tests that the liver enzyme preparations were not required for the metabolic activation of the test compounds. In terms of mutagenic or carcinogenic potency, the aqueous chlorine reaction products were generally higher than the chlorine dioxide fractions except for band #4 of the former fractions (Table 26).

The results of the SCE assays further support those of the Ames assay. These results were as expected since there is good concordance between the Ames and other short-term tests including SCE and chromosomal aberrations (Tennant et al., 1987). In addition to mutagenic activity in the Ames test, byproducts of chlorinated drinking water have been reported to induce both SCE and chromosomal aberrations (CAb) in vitro in cultured mammalian cells (Liimatainen and Grummt, 1988; Meier and Bull, 1985). The disinfectant chlorine itself has been reported to induce CAb in mammalian cells (Mickey and Holden, 1971) while chloramine, administered as the synthetic disinfectant chloramine-T, induced SCE in vitro in cultured

cells (Weitberg, 1987). Chlorinated aquatic humic and fulvic acids have also been reported to induce SCE and CAb. In a study by Meier and Bull (1985), both chlorinated and non-chlorinated humic acids were capable of inducing SCEs in CHO cells. Liimatainen and Grummt (1988) also reported that chlorinated humus-rich surface water induced SCE and CAb in CHO cells *in vitro*. However, contrary to the findings of Meier and Bull (1985), Liimatainen and Grummt (1988) did not detect any SCE or CAb in the non-chlorinated humus samples.

Other chlorination reaction products such as haloaceto-nitriles (HAN) have also been reported to induce SCE *in vitro* in CHO cells. Bull and Robinson (1985) tested the mutagenic and/or carcinogenic effects of five commonly found HAN in water and reported that all produced linear dose-related increases in SCE frequencies. SCE frequencies increased linearly with increasing chlorine substitution and replacement of chlorine with bromine further enhanced SCE induction. The results of the present study confirm others in the literature. The results cannot be directly compared since samples, sample preparation and other parameters were different. However, all the different results point to the fact that genotoxicants are produced as a result of water chlorination. The types and nature of the genotoxicants are varied and diverse and mostly arise due to the reaction of chlorine with different precursor substances.

Identification of the Reaction Products

From the results of the GC/MS data on the liquid-liquid extractions, no chlorinated compounds were detected in the reactions of aqueous chlorine with tryptophan at the 1:1 ratio. The mutagenicity of indolylacetonitrile has been determined in the Ames' test and found to be nonmutagenic (Fielding and Horth, 1986). However, it has been shown to be a precursor of mutagenic activity in cabbage when treated with nitrite (Wakabayashi et al., 1985). There are no other reports in the literature concerning the mutagenicity and/or carcinogenicity of the other indole derivative. The mutagenic precursor activity of norhaman has been determined in the Ames test and reported to possess no such activity (Ochiai et al., 1986). The general mechanism proposed by Dakin (1916) and other workers (Trehy et al., 1986; Le Cloirec et al., 1985; van Temelen et al., 1968) may explain the formation of indolylacetonitrile and some of the products identified in this study. Tryptophan like some other amino acids readily react with chlorine to form mono- or dichloramines which undergo decarboxylation and dehalogenation reactions to form unstable chloroimine derivatives. The intermediates undergo further dehalogenation reactions to form nitriles and depending on the conditions, aldehydes (Fig. 4). Other products are possible from the reactions of tryptophan and chlorine as shown by the general mechanism in Fig. 13.

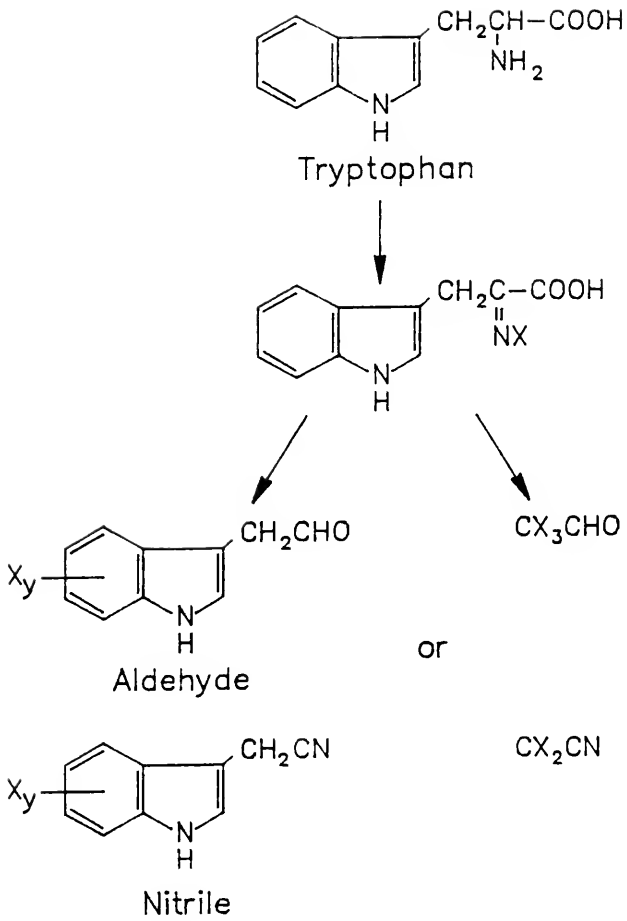

Figure 13. Mechanism for the reaction of tryptophan with aqueous chlorine. X=Halogen. (Modified from Trehy et al., 1986).

When an excess of chlorinating agent was used (7:1 ratio), there were more fragmentation and chlorination reactions leading to the formation of chlorinated compounds. The mutagenic activities of some of the identified compounds such as chloral, 1,1,3-trichloropropanone and 1,1,3,3-tetrachloropropanone have been determined. All three compounds were produced from the reactions of chlorine with humic substances and found to be mutagenic in S. typhimurium TA100 without metabolic activation (Fielding and Horth, 1986; Meier et al., 1986; Coleman et al., 1984; Kringstad et al., 1983). The mass spectra of 1,1,3-trichloropropanone and 1,1,3,3-tetrachloropropanone are presented in Fig. 14. It is not surprising that similar reaction products were formed from the chlorination of tryptophan since there are some structural similarities. The chloroketones are known precursors of chloroform formation and 1,1,3,3-tetrachloropropanone has been identified as a precursor of MX, a very potent bacterial mutagen (Padmapriya et al., 1985; Holmbolm et al., 1984).

The mechanism for the formation of the chloroketones is unclear but it is thought to involve ring chlorination and subsequent ring opening and further chlorination, dechlorination and/or oxidation similar to that proposed by Christman et al., (1983) for alkanoic acids (Fig. 15) or by Rook (1980) for resorcinol (Fig. 16). The mechanism of formation of the quinoline-type compounds is similar to that

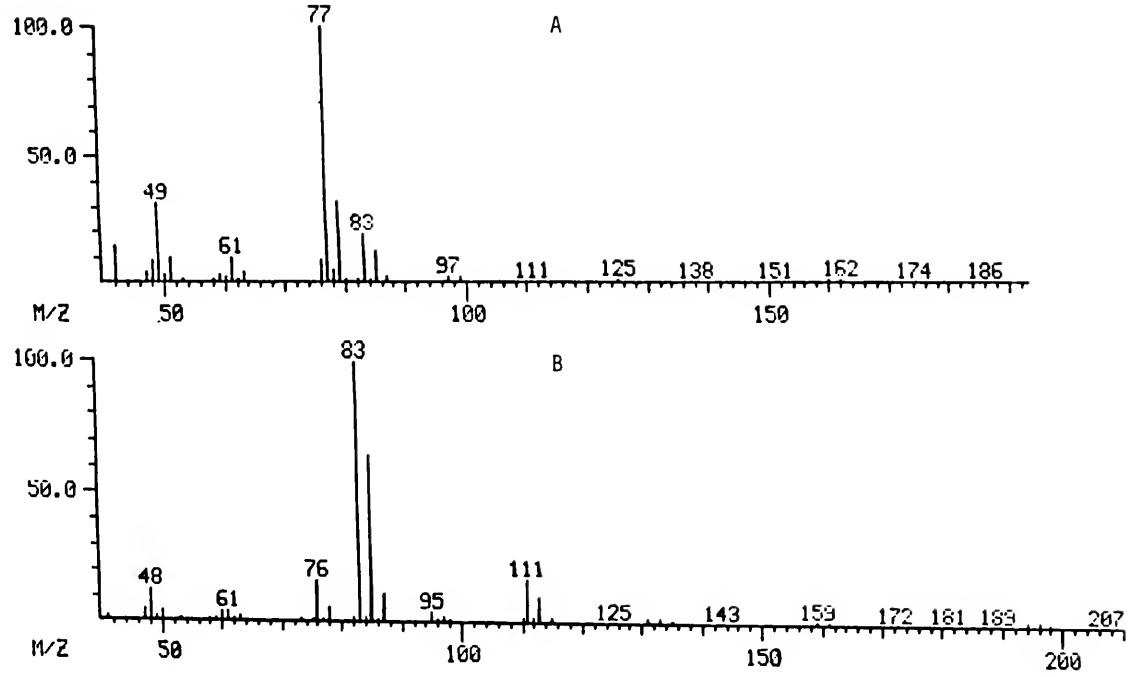

Figure 14. Electron impact mass spectra of A) 1,1,3-trichloropropanone and B) 1,1,3,3-tetrachloropropanone.

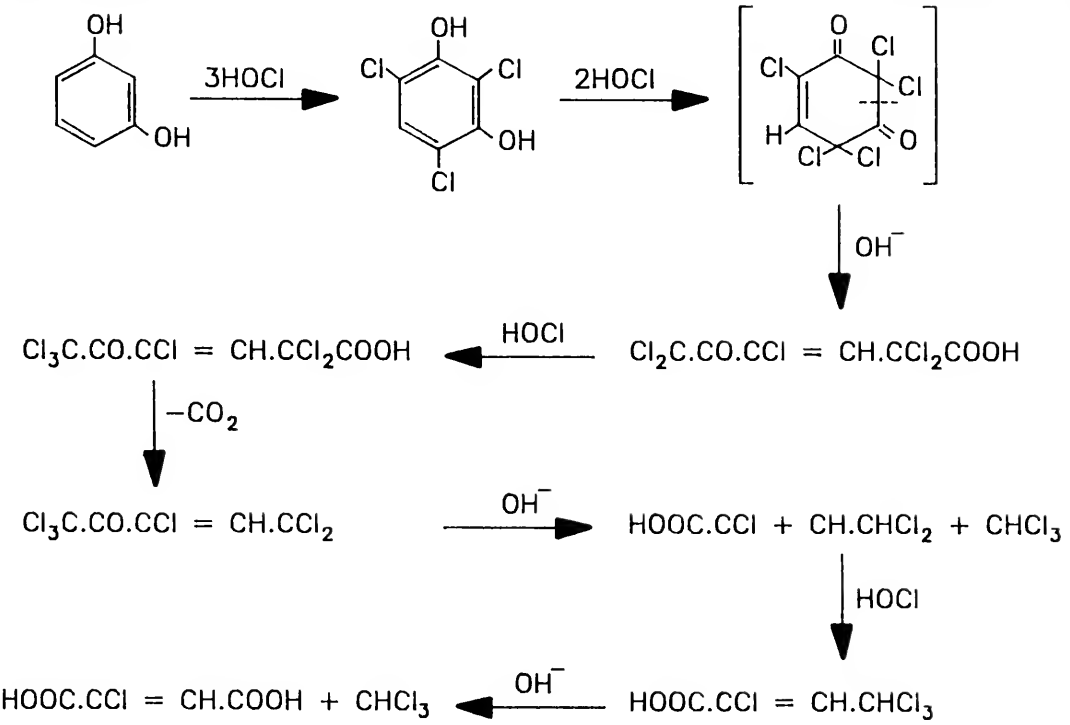

Figure 16. Proposed pathway for the formation of chlorinated acetones and aldehydes from resorcinol (Adapted from Morris, 1978).

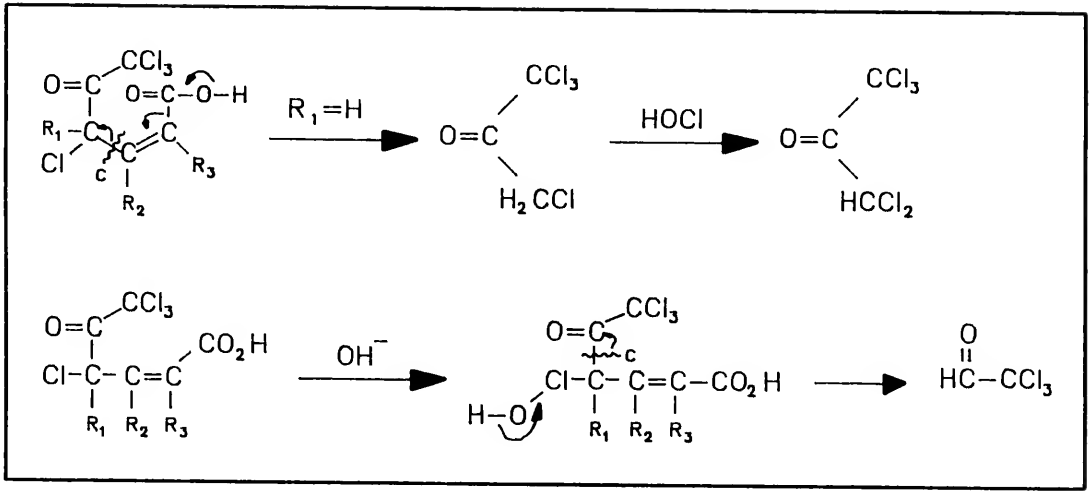

Figure 15. Proposed scheme for the formation of unsaturated alkanoic acids (Adapted from Christman et al., 1983).

of the oxidative decarboxylation reactions reported by van Temelen et al. (1968) (Fig. 17).

Implications of the Mutagenicity to Carcinogenicity of the Findings

Because mutagens have been described as possible carcinogens it is important to determine whether mutagens identified in drinking water pose a health risk to humans. Tryptophan and some of its unstable metabolites such as 3-hydroxykynurenine, have been reported to promote carcinogenesis and increase bladder tumor yields in rats pretreated with a nitrofuran (Friedell, 1977). Treatment of tryptophan with chlorine or chlorine dioxide has been reported to lead to mutagen formation in model reactions (Owusu-Yaw et al., 1989; Sen et al., 1989; Horth et al., 1987). It is also mutagenic upon reaction with nitrite due to the formation of the nitroso derivative (Ohara et al., 1988). The pyrrole ring of tryptophan, like some environmentally significant heterocyclics, is activated and provides sites for chlorination and subsequent haloform formation. Studies by Morris and Baum (1978) indicated that reaction of excess chlorine with tryptophan produced 18% molar yields of chloroform at pH 7.5 compared to almost 100% at pH 11. The prolific chloroform production by tryptophan may explain the formation of the chloroketones identified in this study. The chloroketones are known precursors of chloroform formation in

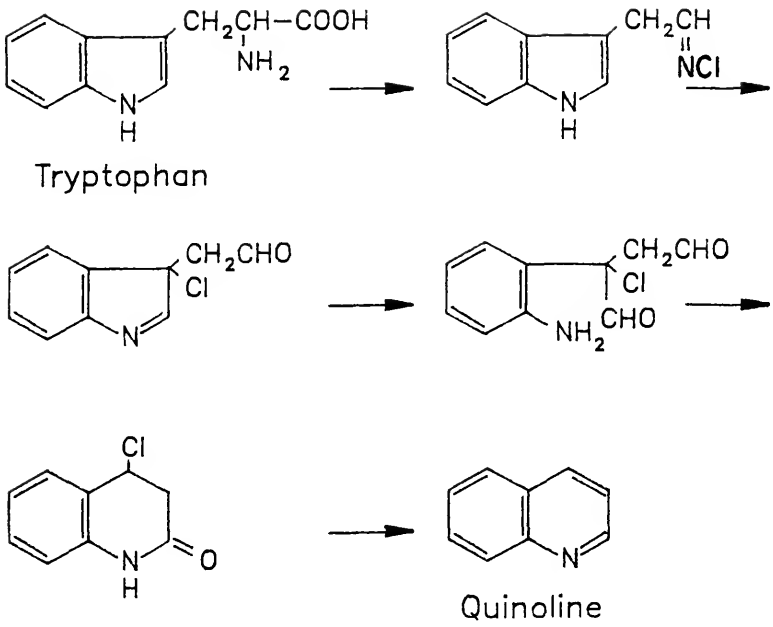

Figure 13. Proposed mechanism for the formation of quinoline from tryptophan (Adapted from van Temelen et al., 1968).

drinking water and since chloroform has already been shown to be a carcinogen, there is still some concern about its production in drinking water.

From the safety point of view, it is likely that some of the identified compounds may contribute to some of the human cancer incidencies worldwide. However, whether some cancers arise solely due to water chlorination has not been proven. Epidemiological data suggest that certain cancers of the colon, bladder and rectum may be due to water chlorination (Cantor et al., 1985; Crump, 1983). It is not possible to substantiate the epidemiological data since they were made after the fact and other factors such as smoking and dietary habits were not accounted for. Even though the organic mutagens are formed in minute quantities their continued presence in drinking water still poses a health problem over the long term. Most of the chlorination reaction products have been tested in bacteria and other mammalian cells but not in humans or whole animals. Attempts at using whole animal assays often prove futile due to the fact that most of the genotoxicants do not require metabolic activation and are metabolized by the enzymes of the liver into inactive forms (Meier, 1988). A solution to the problem is to limit human exposure to these known genotoxicants, find alternative disinfectants, or sorb the organics from drinking after chlorination.

CHAPTER VI
SUMMARY

The mutagenicity data presented in this study indicated that the reaction products from both aqueous chlorine and chlorine dioxide with tryptophan were mutagenic in the Ames Salmonella/microsome assay. The compounds were also capable of inducing SCE in CHO cells. The non-volatile mutagenic reaction products were mainly direct-acting compounds capable of causing both frameshift and base-pair substitution mutations. pH adjustment of the reaction mixture into acidic, neutral and alkaline fractions indicated that neutral and acidic compounds accounted for the bulk of the mutagenic activity. Ethyl ether was found to be more suitable than either methylene chloride or hexane for extracting the chemical mutagens from the aqueous reaction mixtures. The use of Amberlite XAD-2 and XAD-8 resins (1:1 mixture) in one column was found to be more efficient than the use of either resin alone and the order of placement of the resins in the glass column did not have any effect on the amount of mutagens adsorbed on the column. Also, sample acidification to pH 2.5 prior to resin adsorption was found to increase the recovery and subsequent mutagenic activity in the acetone eluates. Amberlite XAD resin adsorption was found to be superior to

liquid-liquid extraction for the concentration of minute quantities of organic mutagens from the model reaction mixtures.

Subsequent TLC separation of the reaction products on silica gel plates resolved the reaction products into identifiable blue and green fluorescent bands. Polar compounds accounted for the bulk of the chemicals present in the TLC subfractions but the strongest mutagens were relatively nonpolar. These results were expected since chemical mutagens are produced in very minute quantities (ppb levels) in drinking water. Further fractionation of a highly mutagenic band on another TLC plate produced one fraction which represented about one-third of total mutagenic activity in the original fraction.

GC/MS analysis of the reaction products indicated that no chlorinated compounds were present at the 1:1 ratio even though some precursors of mutagenic activity were identified. However, at the 7:1 ratio chlorine to tryptophan ratio, there was more fragmentation of the tryptophan molecule leading to the formation of chlorinated byproducts, some of which are known mutagens or precursors of carcinogenic activity.

The health implications of the mutagenic reaction products are not clear since drinking water may contain several chemicals some of which are not produced by chlorination. The results of the present findings are very important especially in the food industry where protein and

amino acid concentrations are likely to be very high. In instances where organic matter in food process water is very high, excess chlorine is used to treat process water for reuse or eventual discharge into the environment (Robinson et al., 1981). Such practices are likely to introduce more mutagens into the environment which may have a serious impact on aquatic life. No studies have been performed under conditions of food processing, therefore the full impact is not known. Also, most of the genotoxicity data on drinking water chlorination byproducts are done in short-term tests which makes extrapolation to risk assessment in humans difficult. The problem may be compounded by the presence of ammonia in the water which may prevent the destruction of proteins. In order to alleviate the problem, the use of coagulation and sedimentation as well as filtration is expected to remove some but not all of the organic matter in drinking or food processing water. Finally, the proposed replacement of chlorine with chlorine dioxide for drinking water disinfection needs to be examined further since both are capable of causing mutagen formation in drinking water.

APPENDIX

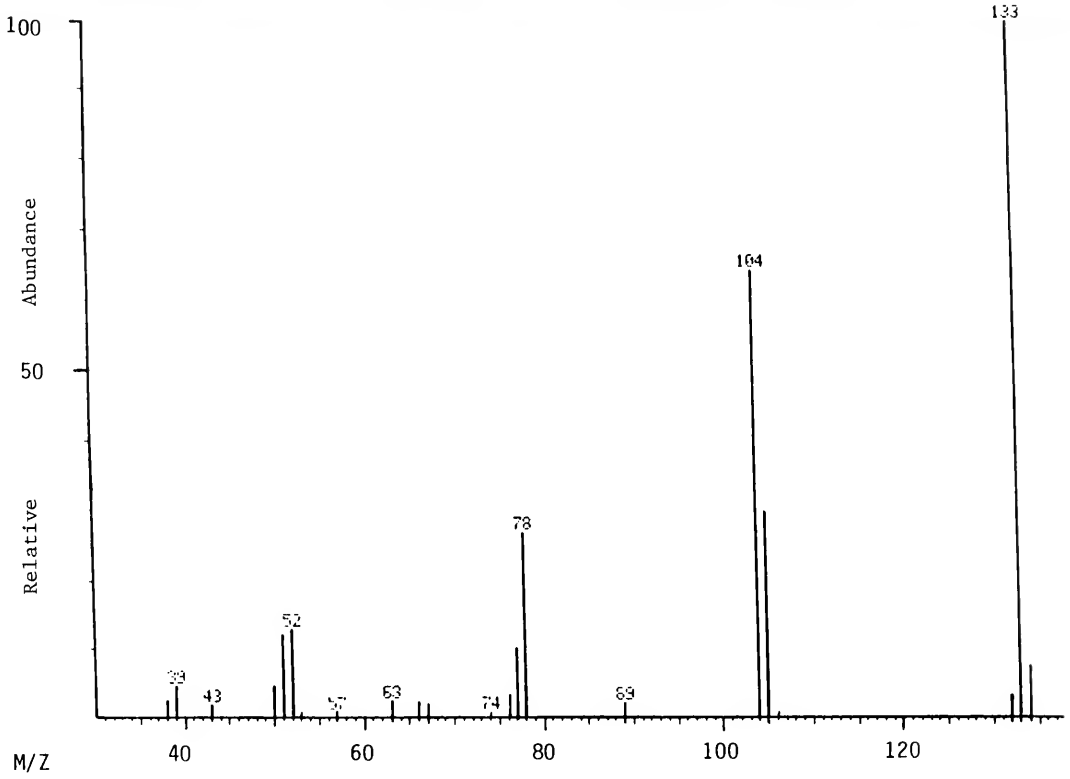

Figure A-1. Electron impact mass spectrum of oxindole.

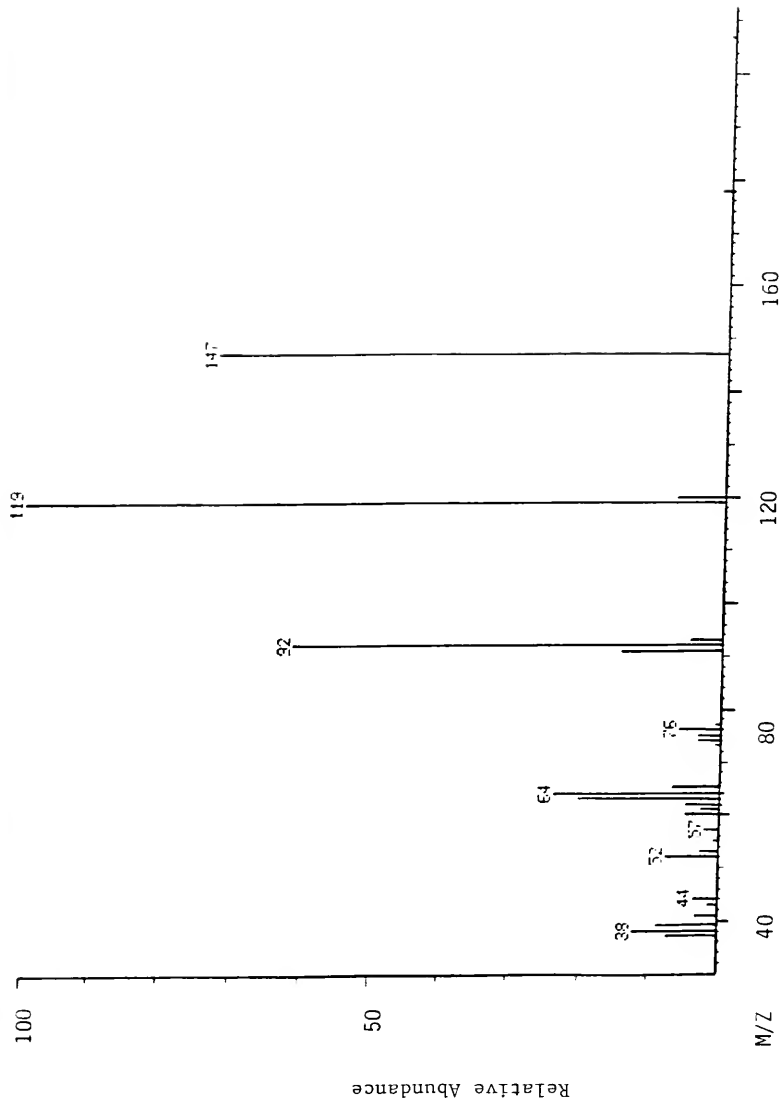

Figure A-2. Electron impact mass spectrum of isatin.

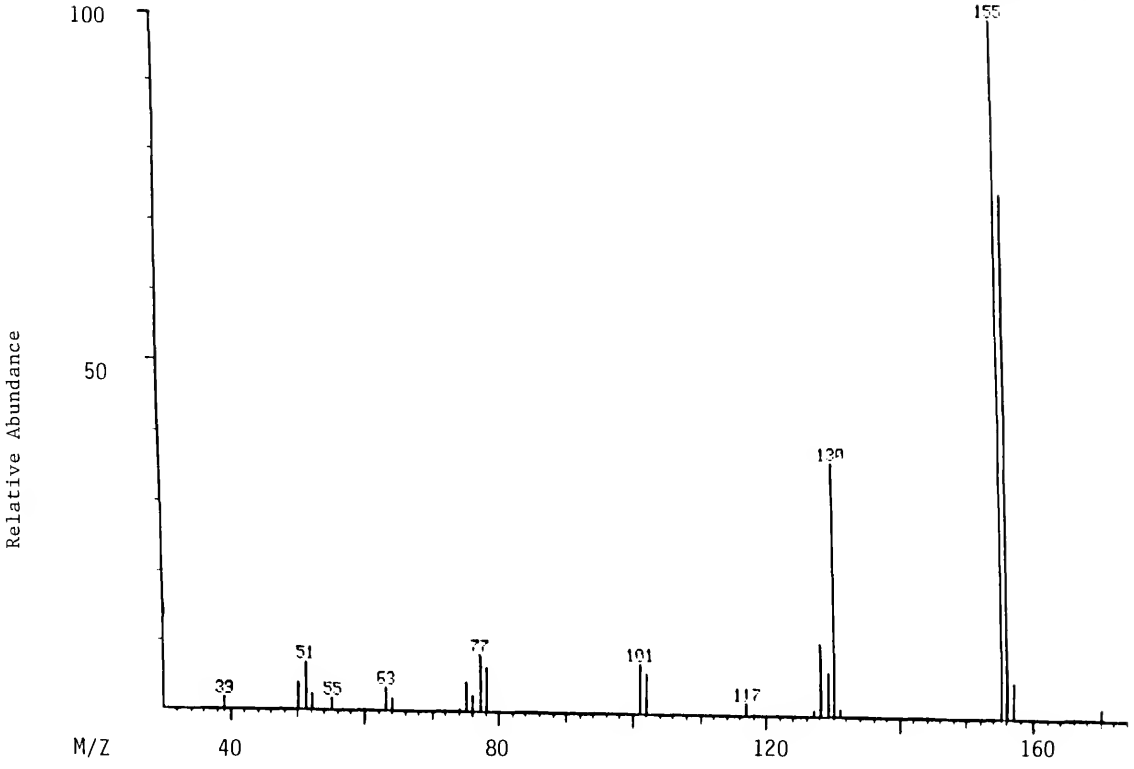

Figure A-3. Electron impact mass spectrum of indolylacetonitrile.

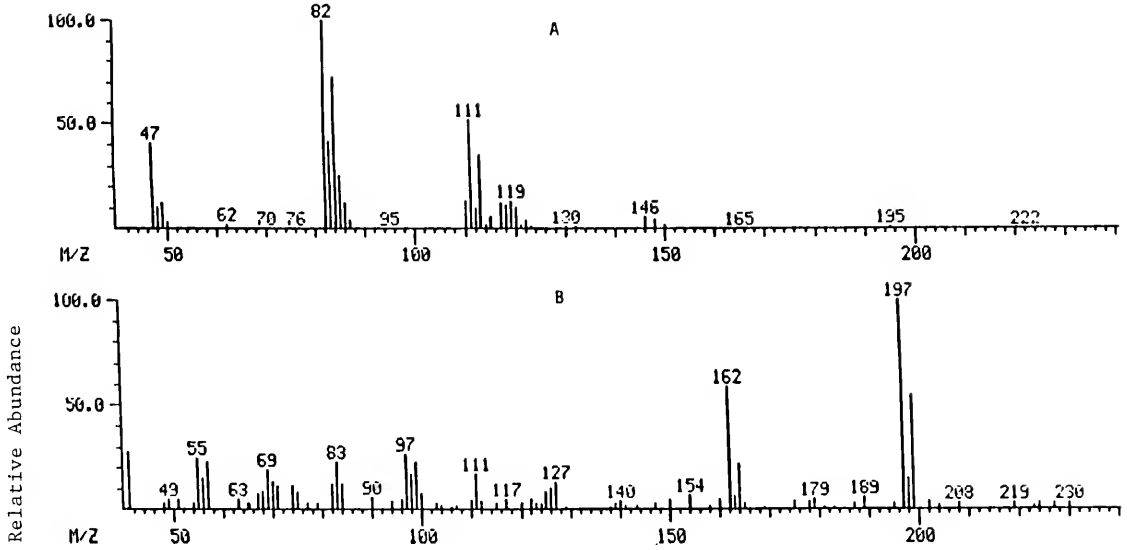

Figure A-4. Electron impact mass spectra of A) chloral and B) dichloroquinoline.

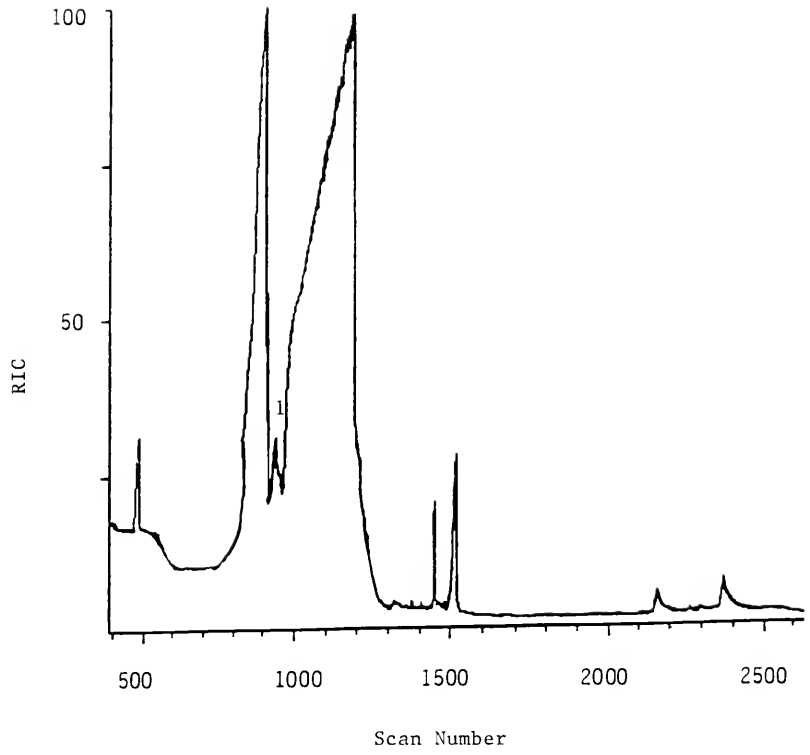

Figure A-5. Reconstructed ion chromatogram of the TLC subfraction of the reaction products of chlorine dioxide with tryptophan (band #4). 1) chloral.

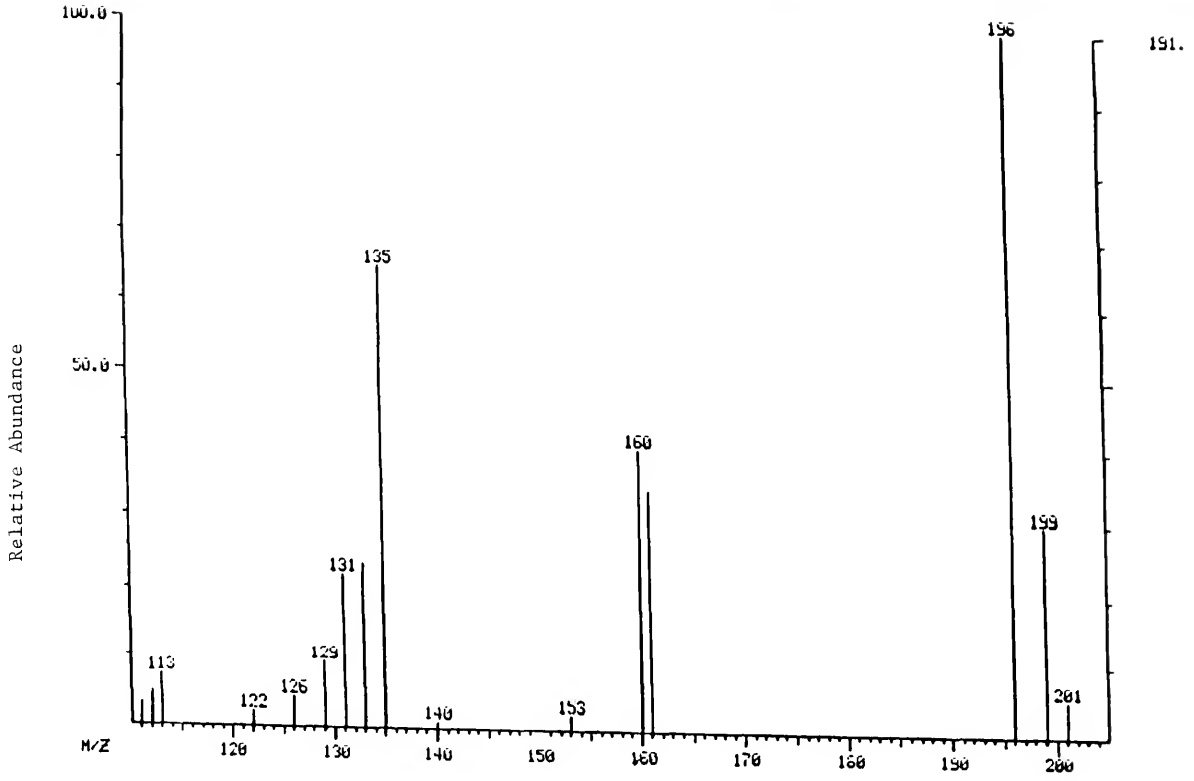

Figure A-6. Chemical ionization mass spectrum of 1,1,3,3-tetrachloropropanone.

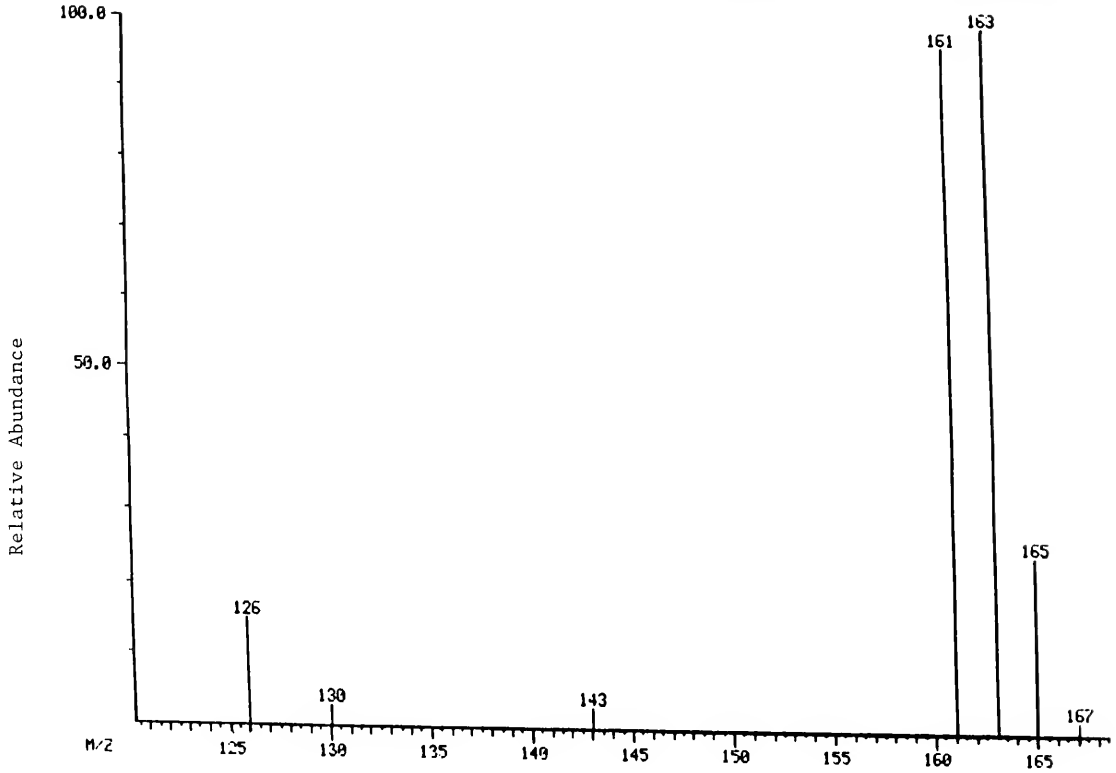

Figure A-7. Chemical ionization mass spectrum of 1,1,3-trichloropropanone.

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BIOGRAPHICAL SKETCH

Joe D. Owusu-Yaw, was born on July 15, 1953, in Kumasi, Ghana. He graduated from Opoku Ware Secondary School, Kumasi, Ghana, in June 1971 and enrolled at the University of Ghana in September of the same year where he received a Bachelor of Science degree with honors in food science and biochemistry in June 1976. After serving a one-year compulsory National Service training at the Takoradi Flour Mill, he took a position as Technical Editor of the Ghana Journal of Agricultural Science. He enrolled at the University of Florida in January 1982 and received a Master of Science degree in food science at the Food Science and Human Nutrition Department in April 1984. He continued his education at the same department and is a candidate for the degree of Doctor of Philosophy to be received in August 1989. He is married to the former Inza B. Gibson and has a son, Tristian, and a daughter, Jocelyn.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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